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ABSTRACT

We isolated rabbit gastric glands to study the actions of various gastrointestinal peptides on the secretion of pepsinogen, intrinsic factor and acid. Oxygen consumption and accumulation of aminopyrine, a radiolabeled weak base, were used as markers for acid secretion in this model. Isolated gastric glands are composed of parietal and chief cells in almost equal proportion, with mucous cells and a few endocrine cells making up the difference. We found evidence for the involvement of both calcium and cAMP as second messengers in the secretion of intrinsic factor and of acid, with synergistic effects from the combination of secretagogues acting via different second messengers. Further analysis of the data reveals a greater acid secretion relative to intrinsic factor secretion for cholecystokinin, a peptide believed to act via the calcium calmodulin second messenger system, than for 8-bromo cAMP and forskolin, activators of intracellular cAMP. Furthermore, histamine, a common secretagoue believed to act exclusively through the actions of cAMP, gave less acid secretion per unit intrinsic factor secretion than any of the other agents. This is evidence that histamine may activate more than adrenyl cyclase when it binds to the cell surface receptor.

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INTRODUCTION

Gastric physiology, like other fields of gastrointestinal physiology, has become increasingly complex as each new technique reveals additional levels of organization in the stomach's response to various stimuli. Until very recently, the vast majority of study on gastric physiology utilized intact organisms. New techniques, especially isolated gastric glands, have revealed that the response of the gastric mucosa in acid, pepsinogen, or intrinsic factor secretion depends upon the interaction of the several different cell types present in gastric pits.

Pepsinogen:

Pepsins are the chief proteolytic enzymes found in human gastric juice. They are stored as zymogens (pepsinogens) in the chief cells of oxyntic gland mucosa and are autocatalytically activated to pepsin by exposure to acid. Pepsins are most active at pH's between 2 and 3.2. In humans, pepsinogen exists as at least two immunologically distinct groups (Pepsinogen I - PGI, and Pepsinogen II - PGII), defined by anodal mobility in agar gel.[127] PGI consists of five components (Pg 1 thru 5) with rapid anodal mobility in agar gel and PGII consists of two components with slower mobility.[128,164] Pepsinogen I is produced only in mucous and neck cells of fundic gland mucosa

while PGII is produced by fundic gland mucosa, the cardiac glands in gastric cardia, pyloric glands in gastric antrum, and by Brunner's glands in the proximal duodenum.[127,134,166] Both are present in serum, although the ratio of PGI to PGII is roughly 6:1.[127] PGI has a molecular weight of 42,500 ± 700 (mean ± SD) daltons, while PGII has molecular weight of 40,520 ± 900 daltons. The difference in size is due to the enzyme moiety as the sizes of the fragments released when the zymogen is activated are almost identical (~5000 daltons).

Pepsinogen Secretion In Intact Subjects:

The first model for the site, storage and release of pepsin was described by Langley in 1881 and was based on light histological examination of fundic mucosa.[104] His model described synthesis of pepsinogen by chief cells, storage in granules, and secretion with stimulation. The great majority of studies on the subject since then have generally studied secretion of pepsin or pepsinogen in live organisms or whole organs. Some of these studies in humans showed that intravenous infusions of secretin stimulated pepsinogen secretion but inhibited gastric acid output during the infusion.[19,25,68,105,110,114,163] Interpretation of the studies was complicated by duodenogastric reflux at the higher doses of secretin. Pepsinogen secretion was also stimulated (~50 percent) in several species (dog, rabbit and rat)

by pentagastrin and gastrin infusions [20,48,163] although in a separate study it was found that higher doses of pentagastrin produced much less than the maximal acid and pepsin.[34] Glucagon infusion shifted the dose response curve for the stimulation of acid and pepsinogen by pentagastrin to the right in this study.[34] Cholecystokinin has been shown to stimulate pepsinogen secretion in dogs.[149] In man, it has been reported to inhibit,[122] stimulate, and have no effect on pepsinogen production.[132] The combination of pentagastrin and secretin increases pepsin secretion more than either alone,[20] while CCK inhibits the stimulation of pepsinogen by pentagastrin in man.[19]

The use of H_2 receptor antagonists cimetidine and ranitidine in the treatment of duodenal ulcers has prompted research into substances that may block basal and stimulated pepsinogen secretion with the hope that this may be of aid in the therapy of both gastric and duodenal ulcers. Omeprazole is a substituted benzimidazole that has been shown to inhibit gastric secretion in man by direct inhibition of the (H-K+) ATPase.[54,108] At high doses (80 mg at night), omeprazole not only shut off acid but pepsinogen secretion as well.[170] The authors postulated that the decreased flow of gastric juice slowed the production of pepsinogen. Hirschowitz et al found that low doses of atropine could effectively shut off both acid and pepsin production a

at doses that yielded minimal, easily tolerable, side effects.[76] By contrast, Aadland and Berstad found no effect from verapamil on the histamine, pentagastrin, or shamfeeding stimulated release of pepsin or acid in humans.[1] The doses they studied yielded no significant cardiac effects.

Proximal gastric vagotomy (PGV) has been found effective in decreasing pepsinogen as well as acid secretion. Haukland, et al, found that insulin-induced hypoglycemia did not induce pepsinogen or acid secretion in patients after PGV.[66] Preoperatively, these patients had shown brisk production of both acid and pepsinogen with this stimulus.[66] Hirschowitz and Helman found that pentagastrin- or histamine-stimulated pepsinogen and acid secretion could be restored in post-PGV patients by cholinergic replacement (i.e., carbamylcholine infusions).[118]

Pepsinogen Secretion In Vitro

The sometimes conflicting and inconclusive data of studies of intact organisms prompted the use of a few *in vitro* models whereby experimental variables might be more easily controlled. Simpson, et al, described the use of isolated frog esophagus to study pepsinogen secretion[143] and Sutton and Donaldson measured the synthesis and secretion of protein, including pepsinogen, by rabbit gastric mucosa in organ culture.[153] These systems

possess several shortcomings in the study of pepsinogen secretion including their complexity and limited opportunity for environmental manipulation. Most of these problems were surmounted in 1977 by the development of isolated gastric glands by Berglindh and Obrink.[11] It is with this model that most recent work has been done. The glands are prepared by collagenase digestion and consist generally of parietal and chief cells with a few endocrine-like and mucus cells. The glands retain cellular connections and are 500 to 1000 um long.[31] Collagenase digestion in the presence of calcium chelators or followed by a calcium chelation step resulted in the preparation of partially purified guinea pig chief[126] and parietal[10] cells, as well as isolated canine fundic mucosal cells[169] but the validity of these preparations has been questioned by at least one investigator. Ayalon, et al, have grown canine mucosal cells in culture and found that they become enriched in chief cells.[8] The degree of responsiveness retained by this preparation has yet to be established.

Studies with isolated gastric glands have yielded a wealth of information about cellular mechanisms of pepsinogen secretion. The glands show a stable, slow rate of basal release and respond to many stimuli with up to 15-fold increase in secretion.[97] Many different secretagogues, including neurotransmitters,

peptide hormones, cyclic nucleotides, and miscellaneous compounds have been found to stimulate pepsinogen secretion. The most accepted are cholinergic agents. Their actions are generally blocked by atropine, indicating action on a muscarinic receptor.[126,97,125] Isoproterenal is also an effective stimulus whose action is blocked by propanol.[97] Stimulation by cholinergic and adrenergic mechanisms appeared to be additive but not synergistic.[97] Surprisingly, histamine, a potent acid secretagogue, did not stimulate pepsinogen release in this study[97] nor in canine mucosal cells.[8]

Several peptides act on isolated gastric glands to produce pepsinogen release. Cholecystokinin (CCK), which as described above, tended to depress or not affect basal pepsinogen secretion in man,[105,122,149] and to inhibit the pentagastrin-stimulated release of pepsinogen in man[19] results in a brisk production of pepsinogen in isolated gastric glands as does a related peptide caerulein.[69,95,125] On the other hand, gastrin has been reported to have little, if any, effect on the production of pepsinogen from rabbit gastric glands.[69,95] The CCK-stimulated production of pepsinogen was inhibited by dibutyryl cGMP.[69,71] Available evidence supports the consideration that gastric cells may contain more than one receptor for the gastrin-related peptides since:

1. Sulfation affects the potency but not the efficacy of

these peptides in their production of pepsinogen, but not of acid.[61]

2. Stimulation of acid by CCK is not blocked by cGMP but the stimulation of pepsinogen is.[69]

Secretin (Koot's preparation) was originally reported to stimulate pepsinogen production but a purer preparation gave no stimulation.[97] Later studies using purified synthetic secretin have demonstrated stimulation of pepsinogen secretion.[69] The same study showed a difference in the time course of pepsinogen stimulation by secretin and carbachol. The rate of secretin-stimulated pepsinogen production was constant over the 20 minutes of the study while carbachol-stimulated secretion was greater in the first 5 minutes tending to plateau after 15 minutes. Furthermore, the combination of carbachol and secretin was found to be synergistic in the stimulation of pepsinogen by gastric glands.[69] Finally, secretin was found to potentiate the actions of acetylcholine on protein secretion from cultured rabbit gastric biopsies.[153]

Vasoactive intestinal peptide has been found effective in stimulating pepsinogen secretion in isolated gastric glands as well,[125] seemingly with the same efficacy as secretin.

At least six agents that modify intracellular cAMP activity have been found to directly or indirectly affect pepsinogen

secretion in isolated gastric glands. Hersey, et al, found that 8-bromo cAMP (8-BrcAMP), dibutyryl cAMP (dBcAMP) and cAMP all stimulated pepsinogen production with a potency sequence of 8-BrcAMP > DBcAMP > cAMP.[71] The efficacies of these substances were equal. The same group found that 8 bromoinosine 3',5'-cyclic monophosphate and forskolin are potent pepsinogen stimulators.[115] Adenosine, adenosine triphosphate (ATP), adenosine monophosphate (AMP), guanosine monophosphate (GMP), 3'5'-cyclic guanosine monophosphate (cGMP), and dibutyryl cyclic GMP (DBcGMP) were all found to be ineffective while 8-bromo cyclic GMP (8 BrcGMP) gave a weak stimulation of pepsinogen production.[71] Several workers found 3-isobulyt-1-methylxanthine (IMX) (an intracellular phosphodiesterase inhibitor) to be active in isolated rabbit gastric glands[69,97,125] and dispersed guinea pig gastric mucosal cells[10] in the stimulation of pepsinogen.

Others have shown that alterations in surrounding pH will stimulate the secretion of pepsinogen. Johnson showed that back diffusion of acid through damaged gastric mucosal barrier in canine Heidenhain pouches elicited a cholinergic reflex stimulation of pepsinogen secretion that may play an important role in ulcerogenesis.[88,89] Acid backdiffusion and gastric intramural pH were subsequently found to be significantly correlated in resting, histamine-stimulated and

metiamide-inhibited rabbit gastric mucosae.[96] Finally, Norris and Hersey showed that small pH changes (from 8.0 to 6.7) in the incubation media of isolated gastric glands gave small additional increases in pepsinogen secretory responses to isoproterenol, carbachol, cholecystokinin octapeptide (CCK-OP), Boots' secretin, and hyperosmolarity. The same pH change gave large increases in pepsinogen secretion when combined with those secretagogues that act to increase intracellular cyclic AMP, i.e., 8-BrcAMP, 8-BrcIMP, and forskolin.[115]

A few other agents have been found to affect pepsinogen secretion in isolated gastric glands. The ionophore A23187 gave a pattern of increased pepsinogen secretion similar to that of carbachol and cholecystokinin in rat gastric glands [125] and guinea pig chief cells.[126] Similarly, addition of 200 mM mannitol to the incubation media of isolated gastric glands resulted in pepsinogen stimulation.[115,78]

Pepsinogen Secretion and Second Messengers

There are few studies of the role of calcium in pepsinogen secretion and most have produced inconsistent data. The pattern and magnitude of the pepsinogen secretion produced by inonphore A23187 was found to be similar to those of carbachol and cholecystokinin.[126,125] Yet, one study found no effect on basal- or CCK-stimulated pepsinogen secretion by the removal of calcium from the incubation medium of isolated gastric glands[95] while

another found a decrease in CCK-stimulated production of pepsinogen by gastric glands in calcium-free medium.[69] No workers have studied whether repeated stimuli in Ca^{2+}-free media will give the same magnitude of response for each stimulus. Calcium-free medium covering canine chief cell cultures decreased their pepsinogen production while chlorpromazine and N, N(-dimethyl) acetyl 3, 4, 5 trimethoxy benzoate (TM B-8), both calmodulin inhibitors, blocked carbachol stimulated pepsinogen release.[131] Culp, et al, found that muscarinic receptors were closely related to guanylate cyclase activity in fractionated glands and that the activity of the guanylate cyclase could be regulated by micromolar changes in calcium concentration, thereby indicating a mechanism for the actions of calcium intracellularly in pepsinogen secretion.[37]

The respective possible roles and interactions of the intracellular second messengers, calcium and cyclic AMP, in the production/secretion of pepsinogen have not been worked out. As above, the calcium ionophore, A23187, gave the same response pattern of pepsinogen secretion in gastric glands as did carbachol and cholecystokinin,[95,125,126] compounds that have been shown to act through calcium/calmodulin systems in pancreatic amylase secretion.[54,168] Similarly, compounds that increase intracellular cAMP actvity, e.g., cAMP, dibutyul cAMP, and 8-bromo cAMP, were shown to generate pepsinogen response patterns similar to those of secretin and VIP.[126,125,95] Combining

these apparently calcium-mediated and apparently cAMP-mediated stimuli (e.g., carbachol and secretin or A23187 and VIP) gave the greatest pepsinogen response (15-fold) of all stimuli in these studies.

Energy requirements for pepsinogen secretion have been little studied in these systems. Kaskebar, et al, showed that CCCP, an inhibitor of mitochondrial energy metabolism, caused a small decrease in basal pepsinogen release and after ten minutes on incubation, abolished the stimulation caused by CCK-OP.[95] Decreasing the incubation temperature from 37 degress to 21 degress to 4 degress Centigrade caused a progressive decrease in basal and CCK-OP stimulated pepsinogen release. There was no detectable stimulation of pepsinogen release by CCK-OP at 4 degrees Centigrade in this study.

Intrinsic Factor:

Intrinsic factor is a glycosylated protein of ~ 44,200 daltons found in normal gastric mucosa and gastric juice that binds cobalamin (vitamin B_{12}) to facilitate its absorption in the terminal ileum.[45,46,41] In humans, the site of intrinsic factor production has been localized to the parietal cells of the body of the fundus of the stomach, the producers of hydrochloric acid.[82-85] Parietal cells synthesize intrinsic factor in rabbits, monkeys, cats and guinea pigs as well.[82,85] In hogs, intrinsic factor production seems to localize to the mucus cells of the stomach pylorus and duo-

denum[83] while intrinsic factor is found mostly in the chief cells of mice and rats. Allen and co-workers have shown using electron microscopy that intrinsic factor is synthesized, as all proteins destined to be secreted, in the endoplasmic reticulum of the parietal cell.[107] The stimuli for the secretion of intrinsic factor will be discussed later.

Castle was the first to propose that the achlorhydria associated with pernicious anemia might indicate that impaired gastric function was somehow responsible for the disease.[129] He found that patients with pernicious anemia responded neither to 200 grams beef nor to 150 milliliters of normal human gastric juice administered daily but that both fed together produced a brisk reticulocytosis. He postulated that an "intrinsic factor" normally present in and secreted by healthy stomachs bound an "extrinsic factor" in beef that together was absorbed and stored in liver as a "hemopoietic factor".[129] Fecal[167] and urinary[135] excretion tests of the physiological handling of radiolabelled cobalamin in pernicious anemics soon supported the gist of Castle's theory.

Cobalamin (vitamin B_{12}) is a large water soluble molecule of 1356 daltons molecular weights that is necessary for the survival of all mammals, but is synthesized only by many microorganisms.[72] Because the molecule is so large, mammals are unable to effectively absorb it passively except in the large doses provided by foods such as liver.[59] Cobalamin exists in nature largely as three forms: (1) methylcobalamin, (2) adenosylcobalamin, and

(3) hydroxycobalamin.[41] Of these, only the first two participate in mammalian biochemistry. Methylcobablamin is a coenzyme in the methyltransferase that shuttles methyl groups from N^5 methyl tetrahydrofolate to homocysteine to form methionine and tetrahydrofolate.[140] Appropriate levels of intracellular tetrahydrofolate are needed for the proper processing of intracellular deoxy uridine monophosphate. It is this defect which is believed to cause the arrest of cellular diversion responsible for megaloblastic anemia. Adenosyl cobalamin participates in the enzymatic reduction of ribonucleotides and is a cofactor in methylmalonyl CoA mutase.[51]

Intrinsic factor will bind all cobalamin derivatives (i.e., hydroxycobalamin, cyanocobalamin, adenosylcobalamin and methylcobalamin) with equal affinity, approximately $10^9 M^{-1}$).[2,3,35,113] This binding occurs over a wide pH range (3 to 9) and the resulting complex is exceedingly stable.[2,3,35,73,74,113,161] Furthermore, the binding of cobalamin to intrinsic factor makes to the complex resistant to pancreatic proteases.[60] However, intrinsic factor binds cobalamin with a considerably reduced affinity at pHs less than three, the pH range of the stomach and early duodenum, and it is believed that cobalamin may initially bind to R proteins. These cobalamin binders are not resistant to pancreatic proteases and are digested to allow intrinsic factor to bind cobalamin.[4] R proteins are a group of cobalamin binders, transcobalamin I, II, and III, found in plasma, salivary secretions and gastric juice that were

originally named for their rapid migration during electropharesis.[4,59] Although intrinsic factor is the most selective, all bind cobalamin tightly.[98] Transcobalamins I and III are glycoproteins of approximately 120,000 daltons molecular weight[5] that seem to function in the blood to carry cobalamin from the terminal ileum to specific receptor sites on liver cells. Transcobalamin II is a small, nonglycosifated protein with an apparent molecular weight of 38,000 daltons[4] that functions in the blood to carry cobalamin from the terminal ileum to cells throughout the body.[45,65,111]

The mechanism of absorption of the intrinsic factor-cobalamin complex in the terminal ileum has been elucidated mostly through the efforts of Donaldson, et al. The binding of the intrinsic factor complex is calcium-dependent, requires no energy, and occurs at a pH between 3.0 and 8.0.[42,43,113] Internalization of the complex (defined as resistance to removal of the complex from terminal ileal cells by calcium chelation or acid wash) requires cellular energy and results in modified intracellular protein with no change in the cobalamin portion of the complex.[81,82] The entire transintestinal transport of the cobalamin requires slightly more than three hours and is dependent upon the expenditure of cellular energy.[83-86] The vitamin is then picked up on the serosal side by the three transcobalamins for transport to the liver and other body tissues.

Intrinsic Factor Secretion In Intact Subject:

The majority of studies on intrinsic factor secretion have been performed on intact subjects. In humans, intrinsic factor secretion, like acid production, is stimulated by histamine,[7,86] pentagastrin[7,160] and cholinergic agents.[160] Basal and pentagastrin stimulated intrinsic factor secretion have been shown to be inhibited by cimetidine in healthy white males[22,50,142] and patients in renal failure.[158] Generally, these studies found that pentagastrin stimulated brisk output of both acid and intrinsic factor and that cimetidine inhibited intrinsic factor output only transiently and to the degree it shut down acid flow, i.e., that the concentration of intrinsic factor in gastric secretion was unchanged with or without cimetidine infusion. Binder and Donaldson found no effect on basal IF secretion and a total block of betazole-stimulated secretion by cimetidine.[22]

One group[52,53] found that cimetidine decreased both the total output and concentration of intrinsic factor in gastric secretion, but that study was flawed in that it compared the intrinsic factor output of two separate groups while the others used each patient as his/her own control. Because intrinsic factor output varies so widely from patient to patient, the decrease in intrinsic factor concentration they found in cimetidine-treated patients could have been due to natural differences between the two groups.

Others have looked at the effects of chronic administration of cimetidine[50,141,142] and ranitidine[172] on the basal and pentagastrin-stimulated output of intrinsic factor. They found not only that intrinsic factor concentration was constant under all experimental conditions, but that parietal cell function returned to normal after only 12 hours withdrawal from H_2 receptor antagonists. This rapid recovery occurred even after 12 weeks cimetidine therapy (1 gm/day).[50] There is no evidence, as yet, of clinical cobalamin deficiency developing as a result of H_2 receptor antagonist therapy.[172]

Somatostatin has been shown by at least two different studies to inhibit the pentagastrin-stimulated secretion of intrinsic factor, pepsinogen, and acid.[159,136] The mechanism has generally been described as a direct competition by somatostatin for the pentagastrin receptor in gastric mucosa[101,136] although the possibility of a secondary inhibition due to somatostatin-induced lowering of splanchnic blood flow was suggested by Wahren and Felig.[162]

Finally, urogastrone has also been shown to inhibit both basal and pentagastrin-stimulated acid production in patients with duodenal ulcers.[105] It also inhibited pentagastrin-stimulated intrinsic factor and pepsinogen increases but had no effect on the basal output of these proteins.

The pattern of intrinsic factor and acid secretion has been the subject of some study as well. In one hour, the pentagastrin stimulated human stomach secretes enough intrinsic factor to bind all the cobalamin present in the average daily diet.[86] Intrinsic factor secretion responds to the same stimuli that generate acid secretion but the times of the peak output of each substance are quite different.[136] Intrinsic factor secretion responds rapidly to most simuli reaching peak within 15 to 30 minutes while acid secretion is still increasing. By the time acid secretion is maximal intrinsic factor secretion has already returned to basal levels.[27] Other studies substantiated these findings.[7,86]

Intrinsic Factor Secretion In Vitro

Intrinsic factor production in isolated glands has been scarecely studied but several workers have used other in vitro models to study the effects of various secretagogues on intracellular messengers.

Experiments with isolated rabiit gastric mucosal biopsies,[93] by Kapadia and Donaldson, found that topical hydrochloric acid induced an increase in pepsinogen secretion but abolished intrinsic factor secretion. The authors postulated that gastric acidfication may block the secretion of intrinsic factor and thus cause the pattern of intrinsic factor and acid secretion seen in previously discussed studies.

Kapadia, Schafer, et al, used isolated rabbit gastric mucosa to study intracellular messengers and found that several potentiators of intracellular cAMP (isobutylmethylxanthine [IBMX], a phosphodiesterase inhibitor, dibutyryl cAMP, and 8-bromo cAMP) all increased intrinsic factor secretion.[92] Histamine increased intracellular cAMP and intrinsic factor secretion and was potentiated by IBMX. Both effects of histamine were blocked by cimetidine. The authors postulated that the intrinsic factor secretion caused by IBMX may have been due to interaction with endogenous histamine since the release of intrinsic factor by IBMX was blocked by cimetidine.[92]

Batzri, et al,[9] used dispersed guinea pig and rabbit mucosal cells to study the relationship between histamine and intracellular cAMP.[9] Histamine increased intracellular cAMP and intrinsic factor secretion as did dibutyryl cAMP. IBMX potentiated both actions of histamine and intrinsic factor secreton by itself. Cimetidine competitively inhibited all effects of histamine in this study.[9] The authors suggested that the binding of histamine with H_2 receptors increases intracellular cAMP to raise the rate of intrinsic factor secretion. Schepp, et al, isolated partially purified chief cells and confirmed that the site of intrinsic factor production in the rat lies in the chief cells, as does pepsinogen, and that carbachol was much more effective in eliciting intrinsic factor secretion than histamine or cAMP analogues.[132,134] The secretion of carbachol-stimulated intrinsic factor was linear over

time in his dispersed rat mucosal cells.[132] Studies by the same laboratory on dispersed human gastric mucosal cells from the resected fundic mucosa of peptic ulcer patients revealed similarities to the same studies in the rabbit,[133] such that secretagogues effective in inducing intrinsic factor secretion were (in order of increasing efficacy): pentagastrin < carbachol < histamine < dibutyryl cAMP < IBMX. The response to histamine was blocked by ranitidine and that of of carbachol by atropine. In both studies, human gastric mucosal cells, when stimulated, secreted a burst of intrinsic factor within 15 minutes of stimulation and continued secreting at a much lower rate than the steady production of intrinsic factor by rat gastric mucosal cells.[132,133]

Acid Secretion:

Golgi first proposed the parietal cell as the source of acid production based on his analysis of histological studies of gastric mucosa close to a century ago.[58] He noted that the parietal cell volume was composed of 30 to 40 percent mitochondria whereas chief and endocrine cells in the same mucosa only contained about 5 percent metochondria. The importance of acid in the causation of peptic ulcer has at least as long a history, reflected in the adage "no acid, no ulcer".[142]

Gastric acid contributes to digestion, with pepsin, in the stomach and is secreted during three physiologic stages of digestion during which a number of stimuli act.[62] Interestingly, pernicious anemics with gastric atrophy and achlorhydria have no clinically detectable digestive difficulties as long as pancreatic function is intact. This observation has prompted some authors to suggest that gastric acid may be more important for disinfection of the gastrointestinal tract than for digestion.

The absolute requirement for gastric acid in ulcerogenesis is undisputed. Menguy[142] reviewed the literature and reported on at least six clinical conditions supporting the requirement for acid for this disease entity:

1. Absence of duodenal ulcer in patients with achlorhydria, as in Addison's disease.
2. High incidence of ulcer in conditions associated with high rates of gastric secretion, such as Zollinger-Ellison and hyperparathyroidism.
3. Healing of duodenal ulcers with reduction of gastric secretory capacity.
4. Production of duodenal ulcers in dogs with esophageal administration of histamine or hydrochloric acid.
5. The formation of a marginal ulcer when an antral remnant is left after subtotal gastrectomy.

6. The fact that duodenal ulcer patients, on average, secrete more acid than ulcer-free patients.

There are four different phases of acid secretion: basal, cephalic, gastric and intestinal.[62] Basal secretion (interdigestive) is that which occurs in the absence of all gastrointestinal stimulation. The degree of basal secretion varies from mammal to mammal. Dogs' basal secretion is generally reported as less than 1 percent of maximal capacity,[140] rats' is about 30 percent of maximal capacity,[123] and man's is about 10 percent of maximal.[172] In both dogs and humans, basal acid may be reduced by vagotomy[6,57] and may be almost abolished by the combination of vagotomy and antrectomy.[26,84] In man, basal secretion shows a circadian rhythm with a high rate in the evening and low rate in the morning. The variation of acid output is not accompanied by corresponding serum gastrin increases.[113]

The cephalic phase is that part of gastric secretion that is evoked by agents from the central nervous system and is eliminated by truncal vagotomy.[62] The primary initiators of the cephalic phase are feeding[119] and interference with supply to the brain of glucose, either by hypoglycemia[24,167] or by infusion of analogs of glucose which cannot be metabolized by the brain (so called "glucoprivation"), e.g., 2-deoxy-D-glucose.[79] Truncal vagotomy abolishes cephalic phase acid secretion in dogs[79] but selective

vagotomy resulted in recovery to about 60 percent of the prevagotomy level at 16 months postoperative.[80] About two years after all forms of vagotomy in humans more than half of the patients had some response, although much smaller than preoperatively to insulin induced hypoglycemia.[90] The acetylcholine secreted by the vagus is believed to contribute to acid secretion in at least two ways:

1. Direct action on the parietal cells.
2. Action on the antrum to release gastrin.

Recent studies have shown that proximal gastric vagotomy abolished the increase in acid and pepsin stimulated preoperatively by insulin-induced hypoglycemia.[66] Hirschovitz, et al, found that the decrement in pepsin and acid secretion in man caused by fundic vagotomy was reversed by cholinergic replacement in the form of urecholine.[77] Furthermore, no amount of pentagastrin infused intravenously could return gastric acid and pepsin output to prevagotomy levels as could the urecholine. Another study in that laboratory found that low doses of atropine could effectively shut down basal acid production in patients with duodenal ulcer at doses that gave less than 20 beats acceleration of heart beat.[76]

Inhibition of acid secretion due to vagal activity has been shown as well. Preshaw showed that although sham feeding induced rises in serum gastrin in dogs with Heidenhain pouches, there was no corresponding stimulation of acid output.[155] He postulated

that vagal stimulation may release an inhibitor that blocks gastrin stimulation of acid. We and others subsequently published studies indicating that the inhibition caused by sham feeding was abolished by truncal vagotomy[124,144] and by resection of the antrum and duodenal bulb.[145] Maximal pentagastrin-stimulated acid secretion in dogs is less than maximal histamine-stimulated secretion. This difference is abolished in dogs by truncal vagotomy, further indicating that vagal activity may inhibit gastrin action. In man, the source of this inhibition has been postulated to be secretin,[121,132] although others believed this effect to be pharmacological rather than physiological.[165] Waldum, et al,[39] found secretin to inhibit gastric acid secretion at a low dose that did not even raise blood serum levels and concluded that secretin may physiologically regulate gastric acid secretion. Their study was complicated by duodenogastric reflux at the higher doses of secretin infusion and the fact that only during the fourth 30 minute period of secretin infusion of was the gastric acid output significantly less than the gastric acid output during the saline infusion.

The gastric phase is so named because the stimuli, distention and the chemical constitutents of food, act in the stomach. Graded distention of the intact stomach of dogs produces moderate rates of acid secretion with a small and ungraded rise in gastrin serum level[152] while graded distention of a vagally innervated antral

pouch produced graded increases in serum gastrin concentration and graded increases in acid secretion.[28] Several common food substances, including caffeine,[28] alcohol,[47,81] calcium ions[81] and protein breakdown products[38,151] are known to stimulate acid production. Caffeine was found to act alone or in concert with histamine to release acid, presumably by inhibiting breakdown of cyclic AMP by cellular phosphodiesterases.[28] Ethanol was found to stimulate acid production when applied in hypertonic solution to dog antral mucosa[47] or when intravenously infused in either dogs[47] or man.[81] Calcium ions in the form of calcium carbonate stimulate both serum gastrin increases and gastric acid secretion in humans.[108] Undigested albumin stimulated no acid production whereas peptic digestion of the albumin made it an effective stimulus. Various amine acids have occasionally been shown to stimulate acid production although only tryptophan has consistently been found effective.[151,100] Both papers on amino acid stimulation of gastric acid secretion suggested that amino acids probably act via nongastrin mechanisms because of the poor correlation between gastrin levels and gastric acid responses. Identification of the gastrin-producing G cells' apical microvillus extending to the gastric lumen has lent support to the theory that food products could act directly to stimulate gastrin release and be modulated by cholinergic input.[148]

Gastrin release is inhibited by acid bathing the antral mucosa, even during sham feeding,[155] antral pounch distention,[39] or concomitant bathing of the antral mucosa with chemical releasers such as liver extract or protein degradation products.[40] However, alkalinization does not release gastrin.[38] Other inhibitory actions on the gastric phase are controversial.

The intestinal phase is so named because the presence of food in the small intestine effects stimuli that regulate digestion. In 1900, it was shown that food in the small intestine stimulated gastric acid output,[106] even when all nerves between the small intestine and stomach were severed. Furthermore, the acid secretion stimulated by infusing liver extract into the small intestine is enhanced by truncal vagotomy.[103] Gastrin is not affected by intestinal meals so that the mechanism of stimulation is unknown although the possible hormone has been named entero-oxyintin.[63]

Acid, fat and hyperosmolar solutions inhibit the intestinal phase stimulation of gastric acid secretion when present in the small bowel. Acid bathing large a surface area of the intestine causes secretin release that blocks further acid secretion.[87] Several peptides have been proposed to be the so-called "enterogastrone" originating in the intestine that acts to block acid secretion when fat is present in the small intestine. One candidate is gastric inhibitory peptide.[120]

Miscellaneous other substances inhibit the secretion of gastric acid in intact humans. The efects of H_2-receptor blockers are well known and therapeutically useful.[22,44] Somatostatin has been shown to inhibit the pentagastrin stimulated gastric secretion of acid in man[136] as has urogastrone.[23] However, verapamil failed to inhibit either acid or pepsinogen secretion in human subjects.[1] The authors tested no other Ca^{2+} channel blockers.

The advent of *in vitro* models, particularly that of isolated gastric glands by Berglindh and Obrink, has allowed for more exacting analysis of the effectors of gastric acid secretion away from the complexity of intact organisms. Glands have been prepared from rabbit, man,[64] rat,[55] and dog.[12] Because gastric glands have proven resistant to cannulating for direct measurement of acid output, acid production by glands can only be measured indirectly. Two common methods have been the accumulation of weak bases and oxygen consumption.[138] Weak bases are unprotonated at physiologic pH so that they can cross biological membranes and are, therefore, protonated and trapped in acid spaces. And, because parietal cells are so rich in mitochondia, any respiratory changes in gastric glands generally reflect changes in parietal cell metabolism.[138,49,

Berglindh, et al,[13] originally showed that ditribyrl cAMP, cAMP and histamine all stimulated respiratory activity and

aminopyrine accumulation while altering parietal cell morphology from typically resting appearance to a stimulated appearance. Carbachol gave a transient increase in both oxygen consumption and aminopyrine accumulation with no changes in morphology. Pentagastrin did not affect any of the parameters. A later study[14] in the same laboratory showed that burimamide (an H_2-receptor blocker) blocked all action of histamine on the glands but did not block the actions of dibutyryl cAMP nor carbachol. Conversely, high concentrations (10^{-4}M) of atropine diminished somewhat the effects of histamine. Thioyanate acted on the glands to decrease the resting aminopyrine ratio from 46 to 2 and totally abolished aminopyrine accumulation in response to all secretagogues, despite stimulation of O_2 consumption.

Work in other laboratories since then has tended to support original work in Berglindh's laboratory. Chew and Hersey found a small, cimetidine-resistant, stimulation of oxygen consumption and aminopyrine accumulation by gastrin in isolated rabbit gastric glands.[33] To measure the response required, the inclusion of either dithiothreitol or isobutyryl-1-methyl-xanthine. Other workers have been unable to detect a response to gastrin in isolated glands[168,169] and have tended to support hypotheses that gastrin

either acts to release histamine (in the rabbit at least) or that its binding to the parietal cell potentiates the action of histamine.[15,18,33]

Several studies have shown that histamine acts to increase intracellular cAMP in rabbit gastric glands prior to stimulating aminopyrine accumulation and oxygen consumption consistent with its acting as a second messenger for histamine.[15,16,32,33] Furthermore, analyses of ED_{50} (dose required to give 50 percent maximal response) for histamine and pA_2 values for cimetidine showed close similarity for respiration, aminopyrine accumulation, adenylate cyclase activity and cAMP accumulation.[32]

The thrust in our work has been to contrast all the different response parameters of the glands - pepsinogen secretion, intrinsic factor secretion, aminopyrine accumulation and oxygen consumption - in order to better understand the functioning of the gland in its entirety. Various studies have shown interactions between endocrine cells and the other cell populations in the glands and it is reasonable to hypothesize that there may be intercellular communication among the cells of the gland as well. Finally, we have begun to analyze the intracellular proteins that are phosphorylated in response to intracellular second messengers.

MATERIALS

We used 3 to 5 kilogram male New Zealand white rabbits to prepare gastric glands as described by Berglindh and Obrink. Collagenase type 1, soybean trypsin-innibitor, carbamylcholine, forskolin, rabbit albumin, bovine albumin, A23187 bovine hemoglobin, 8-bromo-cAMP, histamine and Folin's reagent were all obtained from Sigma.

Cholecystokinin-octapeptide (sulfated), pentagastrin and bombesin were all obtained from Peninsula Laboratories.

Trowel's medium was obtained from Gibco. Charcoal (Norit "A") was also obtained from Sigma.

All other chemicals used were of the highest available purity.

^{57}Co-Cbl (Lot 201) was obtained from New England Nuclear; 0.2 ml ^{57}Co-Cbl (0.7 ug/ml) was diluted to 25 ml to give a 5.54 ng/ml solution with ~12,000 counts/min/ml.

A human intrinsic factor standard, obtained by gastric suction exactly two hours after pentagastrin stimulation, was graciously donated by Dr. Cyrus Kapadia.

Solutions:

1. Phosphate-buffered saline (PBS) was made according to the following specifications and adjusted to a pH of 7.3:

NaCl	149.6 mM
K2HPO4	3.Ø mM
NaH2POuHoO	Ø.64 mM

2. The collagenase digestion medium was made according to the following specifications:

   a. Precollagenase solution:

NaCl	13Ø mM	stored at 4^{o}C.
NaH2PO4.H2O	3 mM	
Na2HPO4	3 mM	
K2HPO4	3 mM	
MgSO4.7H2O	2 mM	
CaCl2.2H2O	1.Ø mM	
phenol red		

   b. Final collagenase solution:

      (1) Bicarbonate solution 252 mg Na HCO3 dissolved in 25 ml H2O.

      (2) To make solution A, mix 5Ø millitiers of precollagenase solution, 1Ø milliliters bicarbonate solution and 4Ø militers of H2O in a 1ØØ milliliter volumetric flask.

(3) The final collagenase solution consists of the following ingredients dissolved in 50 milliliters of Solution A:

Collagenase	50 mg
Soyvbean trypsin inhibitor	5 mg
Rabbit albumin	50 mg
Dextrose	100 mg

c. Solutions for pepsinogen assay:

(1) Dilute hydrochloric acid for pepsinogen activation.

16 ml 0.04 N HCl

25 ml 0.20 N KCl

Dilute to 100 ml with distilled H2O

Adjust pH to 1.8 with 1 N hydrochlorine acid

(2) Hemoglobin solution for pepsinogen digestion substrate.

Dissolve 2 g of bovine hemoglobin in dilute hydrochloric acid solution above

(3) 5% TCA (v/v)

Mix 5 cc 100% TCA in 95 cc H_2O

d. Solutions for intrinsic factor assay:

(1) Hemoglobin-coated charcoal:

Dissolve 0.25 g bovine hemoglobin in 50 ml H2O

Suspend 2.50 g charcoal ("Norit A") in 50 ml H2O

Slowly pour the two solutions together, swirling constantly

METHODS

A. Harvesting The Glands

Male New Zealand white rabbits, 7 kg, were used to prepare gastric glands as described by Berglindh and Obrink.[16] the rabbits were anesthetized with 2.5 to 3.0 ml of sodium pentobarbital (50 mg/ml) injected into a lateral ear vein with a 26 gauge needle. The abdomen was shaved and an additional 0.5 to 1.0 ml of sodium pentobarbital was administered as necessary for adequate anesthesia.

B. The Procedure.

The rabbit was immobilized in the supine position and a midline abdominal incision was made to expose the abdominal contents. The pedicle of the superior mesenteric artery was isolated, as were the right and left renal arteries. The abdominal aorta was exposed with blunt dissection and tied off proximal to the iliac bifurcation with a silk tie. A bulldog clamp was placed on the abdominal aorta just distal to the renal arteries. Clamps were also placed on each renal artery. An aortotomy was made proximal to the ligature and the aorta was cannulated in a retrograde fashion with a pediatric cardiac catheter. The bulldog clamp was removed and 1.5 cc sodium heparin was injected and

flushed with 25 ml of phosphate buffered saline (PBS). The heparin was allowed to circulate for two minutes. Following this, a left thoracotomy was performed with heavy scissors from the xyphoid process to a point just inferior to the axilla. The rabbit was exsanguinated through the aortic cannula, the thoracic aorta clamped, and a portal vein venotomy was performed. Two litres of warmed, oxygenated PBS were perfused through the aortic cannula in a retrograde fashion, with a roller pump perfusion apparatus at 40 ml/min. Following the perfusion, the stomach was removed, the antrum discarded, and the stomach opened along the greater curvature. The gastric contents were emptied, the stomach rinsed in cold PBS, cleaned with cotton, and then blotted on dry filter paper to remove as much gastric contents and mucous as possible. The mucosa was then bluntly separated from the submucosa with a scalpel handle and minced into small pieces. A wet weight was obtained.

The minced mucosa was then placed into a collagenase solution of 100 units/ml with a gas phase of 95% O_2 and 5% CO_2 and incubated at 37 degrees C. in a shaker bath (60 oscillations/minute) for 60 minutes.

Following this, the solution was filtered through three successively small pored nylon cloths. The glands were washed three times in approximately 200 ml of Trowel's medium. After each wash, the glands were allowed to settle and the supernatant was aspirated. The glands were then resuspended in approximately 125 ml of Trowel's medium and placed back into the shaker bath to be oxygenated with 95% O_2/5% CO_2.

After the final equilibration, the glands were placed in a beaker and stirred with a magnetic stir bar and 1.8 ml of gland suspension was pipeted into a 50 cc screw top culture tube containing with 0.2 ml of secretagogue for experimental tubes or 0.2 ml media for control tubes. The experiments were additionally controlled by designating four tubes each experiment as "zero time" tubes and three tubes were filtered immediately without incubation. The tubes were immediately gassed with 95% O_2/5% CO_2 and were regassed at 15 minute intervals. A millipore vacuum filter system and fiberglass prefilters were used to separate glands from incubation medium at the end of 30 minutes. The supernatants were then assayed for pepsinogen and intrinsic factor.

C. Aminopyrine Accumulation

Aminopyrine (AP) is a weak base that will accumulate in intracellular acid spaces after accepting a proton but, in undissociated form, rapidly crosses biological membranes. It was originally used in isolated gastric glands by Berglindh.[2,3] We used radiolabeled ^{14}C-aminopyrine and calculated the ration of intraglandular to extraglandular aminopyrine according to the following equation:

$$\frac{\text{AP in intraglandular water}}{\text{AP in respiratory medium}} = R = \frac{1-10^{(pKa-pHgland)}}{1-10^{(pKa-pH\ medium)}}$$

where R is the accumulation ratio, pKa is the dissociation constant of aminopyrine, pHgland is the average pH in the intraglandular water, and pH medium is 7.4.

Isolated gastric glands were obtained as described above except respiratory medium (composition outlined in Methods) was used, and 0.8 uCi/ml ^{14}C-labelled

aminopyrine was added to the final washed gland suspension. The secretagogues were dissolved directly in the medium and a total incubation time of 60 minutes was used. At appropriate time intervals, 1 cc of the gland suspensions were transferred to 1.5 cc microfusion tubes and in a Beckman microfuge at 2000 g for 1 minute; 0.5 cc of supernatant was removed and the pellet washed in fresh incubation media and remicrofuged. The supernatant was withdrawn and discarded and then concentrated HNO_3 and 300 ul of distilled H_2O was added to each tube. 0.5 cc samples were counted in a Beckman liquid scintillation counter with automatic quench control. In addition, 0.5 cc of supernatant was counted. The obtained counts were used to calculate R for unstimulated and for secretagogue-stimulated glands. Results were expressed as percent of unstimulated $\pm$ standard error of the mean. Differences between means were tested for significance using Student's T test for paired and unpaired data.

D. Oxygen Consumption

Isolated gastric glands were prepared as described above except that the medium used was respiratory medium (compostion described in Materials). Oxygen comsumption was studied at 37 degrees C. in a Yellow Springs Instruments O_2 consumption apparatus with a Clark-type polaragraphic probe. After equilibration, 3.0 cc of gland suspension was added to 3.0 cc of media or secretagogue. Saturation measurements were obtained at 5 minute intervals for 30 minutes. The respiratory activity was recorded as ul O_2/min. Results were expressed as percent of unstimulated $\pm$ standard error of the mean. Statistics were performed as described above.

E. Pepsinogen Assay

Pepsinogen was assayed by a modification of the method of Anson and Mirski.[5] After filtration, 0.1 ml of each sample was placed in a 16x125 mm test tube. Two 1 ml samples of the final washed gland preparation were frozen in dry ice and then thawed; 0.1 ml of each sample was used to determine the total pepsinogen of

the preparation. Ø.3 ml of an isotonic KCl solution with pH adjusted to 1.8 was added to each tube to contnate the pspsinogen. The tubes were vortexed and allowed to react for ten minutes in a shaker bath at 37 degrees C. Then Ø.8 ml of 2% bovine hemoglobin in the same pH-adjusted KCl solution was added to each tube and the tubes re-vortexed and placed into the shaker bath for an additonal 4Ø minutes. The samples were removed, 1.8 ml of 5% TCA was added to each tube, and the samples were gravity-filtered through grade 4 filter paper. After ten minutes, 1 ml of each sample was removed and placed into fresh test tubes. Five ml of Ø.2 N NaOH was added to each sample and allowed to react for ten minutes. Then Ø.5 ml of 1 N Folin's reagent was added to each tube. At the end of 3Ø minutes, the samples were read for optical density at 76Ø nm on a Beckman's spectrophotometer. A peptic unit has been defined as that amount of pepsin which releases Ø.1 mole tyrosine from 5.Ø ml 2 percent hemoglobin at pH 1.7 in 1Ø minutes at 37 degrees Centigrade. Workers in our laboratory have established that value for peptic units can be obtained by multiplying the optical density of the sample at 76Ø nm by the factor 47.8 and then multiplying by any dilution

factor. A) The relationship between optical density and peptic units in our standard assay is illustrated in Figure 1. The conversion from optical density to peptic units was performed as explained in the legend for Figure 7. The results were expressed as a percent of total pepsin activity according to the following equation:

$$x = \frac{x' - y}{t}$$

where: x' = raw value

y = zero time value

t = total pepsinogen

Work in our laboratory has demonstrated a tendency for a rising baseline throughout the course of the experiment, i.e., that control tubes begun at the end of the experiment contained more pepsinogen than those begun at the start. Several studies in our laboratory indicated that these changes were linear with respect to time. Therefore, results for each experiment have been corrected for this change by a linear amount calculated by the change in control tubes from the begin-

ning to the end of the experiment to prevent any anomalous results. Statistics were performed as described above.

F. Intrinsic Factor Assay

We used a method first developed by Gottlieb et al in 1965.[130] In 15 ml plastic conical centrifuge tubes, we mixed 0.5 ml sample, 2.5 ml of 0.9% NaCl (pH 7.4)-BSA and 0.2 ml ^{57}Co-cyanocobalamin. The system was allowed to react for 30 minutes and then 2 ml of hemoglobin-coated charcoal prepared as described above was added and reacted for 20 minutes. The tubes were then centrifuged at 3000 rpm for 20 minutes. Four ml of supernatnat was removed and placed in scintillation tubes. Both the pellet and the supernatant were counted in a Beckman gamma counter. Results were expressed as percent of basal (unstimulated). Statistics were performed as described above.

RESULTS

I. CHOLECYSTOKININ

A. Aminopyrine Accumulation:

We chose a concentration of 10^{-6} M cholecystokinin. The development of aminopyrine accumulation over time is shown in Figure 2. The differences were significant to at least $p<0.05$ at all time points tested, indicating stimulation of acid production as early as 5 minutes, with a plateau at 45 minutes.

B. Intrinsic Factor Secretion:

The maximal secretion of intrinsic factor by cholecystokinin was 130$\pm$ 5 percent over unstimulated at a secretagogue concentration of 10^{-6} M. The differences between stimulated and unstimulated were statistically significant at all doses with $p<0.05$. The dose response curve is given in Figure 12e and the time response in Figure 12c. Over time, cholecystokinin released intrinsic factor in a linear fashion that began to plateau at 25 minutes. Unstimulated secretion of intrinsic factor was essentially linear throughout the 60 minute time period of the experiment with a rate of secretion of intrinsic fact of ~ 4000 counts per minute.

C. Pepsinogen Secretion:

CCK-OP stimulated a large output of pepsinogen, up to 390 percent of basal output. The dose response curve is given in Figure 3. This maximal secretion occurred with a CCK concentration of 10^{-7} M; 10^{-6} M concentration gave a lesser stimulation than did 10^{-7} M or 10^{-8} M. The stimulation was essentially linear from 10^{-9} M to the peak stimulation.

II. HISTAMINE

A.Aminopyrine Accumulation:

The curves for histamine-stimulated and unstimulated aminopyrine accumlation with respect to time are shown in Figure 2. The maximal stimulation by histamine at 30 minutes was 130$\pm$ 3 percent over unstimulated. The data shows a gradual stimulation of acid secretion over time, approximately linear, until 30 minutes when the accumulation levels off and falls over the longer time periods. This falloff is likely due to metabolism of histamine by the glands, since they have been shown to have that capability.

B. Oxygen Consumption:

Figure 4 shows the several different runs of oxygen consumption by unstimulated and histamine-stimulated glands. The concentration of histamine was 10^{-5} M in the stimulated runs and each set represents a different comparison. (The oxygen consumption by unstimulated glands indicates that all gland preparation were viable.) The oxygen consumption at 10^{-5} M histamine at 60 minutes was 40 percent desaturation on one run and 36 percent desaturation on the other.

C. Intrinsic Factor Secretion:

The dose response curve for histamine-stimulated intrinsic factor secretion is given in Figure 12a and the time-response curve in Figure 12a. A concentration of histamine of 10^{-4} M gave the greatest stimulated intrinsic factor release at 30 minutes of 48$\pm$ 4 percent over basal. All concentrations gave significant differences when compared to unstimulated values ($p<0.05$).

Histamine-stimulated secretion of intrinsic factor is linear with respect to time up to 60 minutes. There was no initial burst of intrinsic factor release. The difference between stimulated and unstimulated was not significant until 10 minutes ($p<0.05$). All subsequent time periods gave strongly significant stimulation.

D. Pepsinogen Secretion:

The dose response curve for histamine-stimulated pepsinogen secretion is shown in Figure 3. Only the two higher concentrations of histamine (10^{-4} M and 10^{-3} M) stimulated any significant pepsinogen secretion and that was only 32± 5 percent over unstimulated (basal) at 10^{-3} M.

III. 8-BROMO-cAMP

A. Aminopyrine Accumulation:

We studied aminopyrine accumulation in isolated gastric glands using 10^{-4} M 8-bromo cAMP. Figure 2 shows the accumulaton curve with respect to time. The accumulation is linear from 5 to 30 minutes when it plateaus. All points are significant compared to basal or unstimulated at $p<0.05$.

B. Oxygen Consumption:

We used $4x10^{-3}$ M 8-bromo cAMP to evaluate oxygen consumption by gastric glands with this secretagogue. The oxygen desaturation curve is shown in Figure 6. By 35 minutes, essentially all available oxygen had been consumed by the glands. The differences between stimulated and unstimulated were significant at all time periods from 15 minutes onward.

C. Intrinsic Factor Secretion:

At 60 minutes, 8-bromo cAMP (10^{-4} M) resulted in 83$\pm$ 2 percent over unstimulated secretion of intrinsic factor. The difference was statistically significant at $p<0.05$. The dose response curve is shown in Figure 12b. The secretion with respect to time is shown in Figure 12b. The secretion was linear from 0 to 30 minutes when it plateaued. From 5 minutes onward, the difference was statistically signification ($p<0.05$).

IV. FORSKOLIN

Forskolin is a diterpene originally isolated from the Indian plant Coleus forskohtii[172,173] that has been shown to activate adenylate cyclase in cellular homogenates and to increase intracellular cAMP accumulation in intact cells.[174,175]

A. Aminopyrine Accumulation:

Figure 2 gives the curve of aminopyrine accumulation with respect to time for glands treated with forskolin at a concentration of 10^{-4}M, a dose shown to give maximal animopyrine accumulation in other studies in our laboratory. Results are consistent with an early burst of acid secretion within the first 15 minutes and a slower, approximately linear, rise over the next 45 minutes to a peak aminopyrine accumulation of 328± 18 percent over unstimulated accumulation at 60 minutes. This difference was statistically significant at $p<.001$.

B. Oxygen Consumption:

Forskolin at 10^{-4} M stimulated a brisk oxygen consumption that, in the stimulated flasks, essentially consumed all the available oxygen present in the medium by 30 minutes. Curves are shown in Figure 5. At 30 minutes, the stimulated oxygen consumption was 265± 14 percent of unstimulated consumption, statistically significant at $p<0.05$.

C. Intrinsic Factor Secretion:

The maximum secretion of intrinsic factor was 85± 2 percent over basal at 45 minutes as shown in Figure 12d. This was statistically significant at $p<0.05$.

The stimulation with respect to time revealed a burst of secretion by 10 minutes that essentially plateaued from then to the end of the experiment at 60 minutes. From 10 minutes onward, the slope of the stimulated curve was significantly less than of the unstimulated, perhaps indicating a depletion of intracellular intrinsic factor and secretion at a slower rate constrained by the rate of synthesis of intrinsic factor. The rate of unstimulated release was significant from the beginning to the end, as in other experiments.

D. Pepsinogen Secretion:

Forskolin stimulated a peak pepsinogen secretion of 14.2$\pm$ 1.9 percent of total, almost five-fold the unstimulated secretion. The amount released plateaued at 10^{-5} M and tailed off at 10^{-4} M. The differences were statistically significant $p<0.05$. The dose response curve is shown in Figure 3.

V. COMPARISONS AMONG SECRETAGOGUES

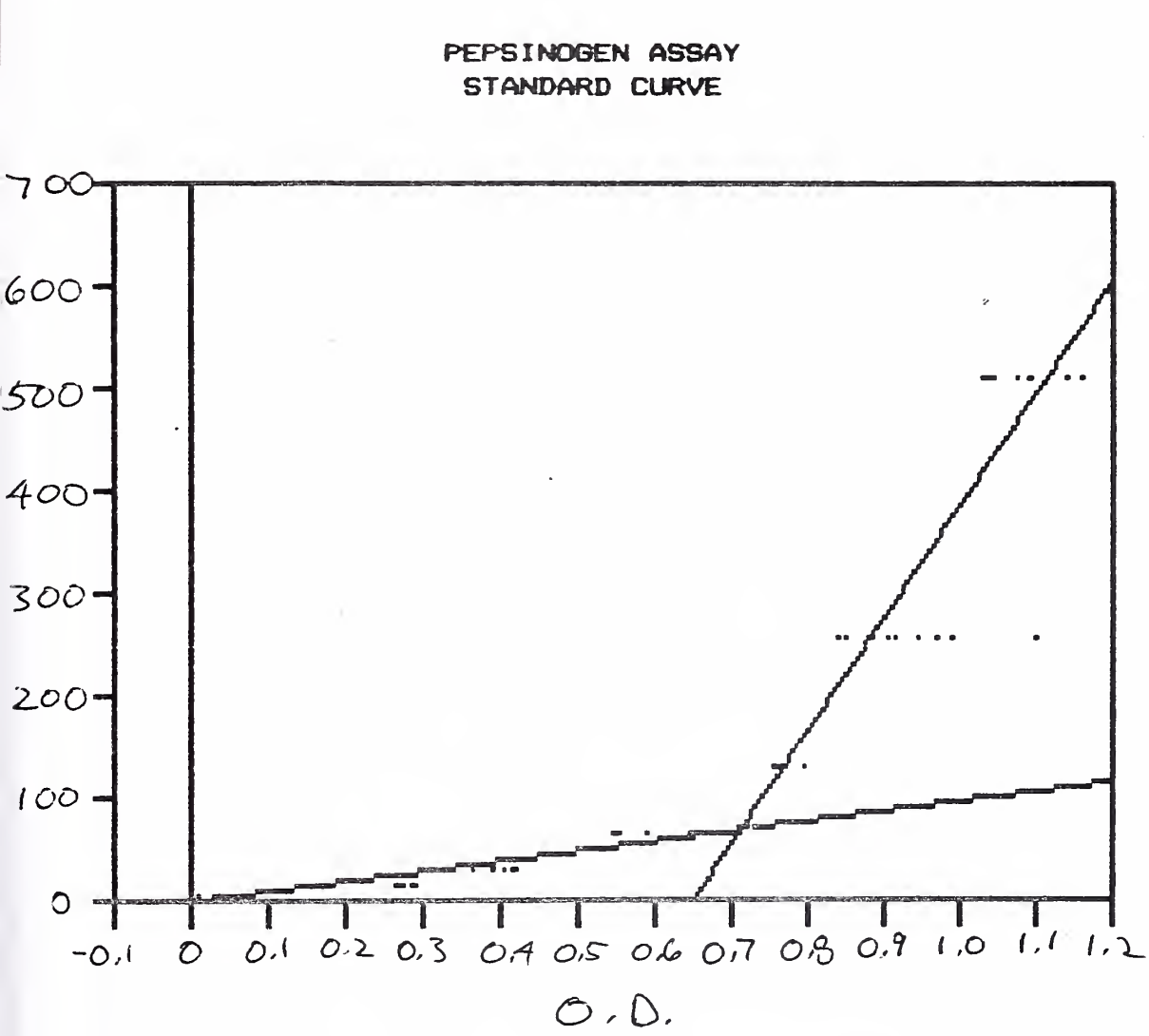
Figures 7-10 illustrate comparisons among secretagogues of relative efficacy in stimulation of aminopyrine accumulation, intrinsic factor secretion, and oxygen consumption. Table 1 gives the equations generated by linear regressions of the points appropriate for each comparison and the correlation coefficients thus derived.

A. Intrinsic factor secretion versus aminopyrine accumulation cholecystokinin showed relatively much greater aminopyrine accumulation per unit intrinsic factor secretion than the other secretagogues. Histamine showed the least while 8-bromo-cAMP and forskolin were intermediate. Their actions were statistically similar. The observed differences between the histamine and 8-bromo cAMP/forskolin lines are interesting since histamine is believed to act via the cAMP system, as do 8-bromo cAMP and forskolin.

B. Aminopyrine accumulation versus oxygen consumption Figure 8 gives the curves for comparison of aminopyrine accumulation and oxygen consumption between histamine and 8-bromo cAMP/forskolin. Histamine required considerably greater oxygen consumption per unit aminopyrine accumulation than 8-bromo cAMP and forskolin. Again, this is surprising given the accepted commonality of their believed intermediates.

C. Intrinsic Factor Secretion Versus Oxygen Consumption.

Figure 9 gives the curves for comparison of intrinsic factor secretion versus oxygen consumption for histamine and 8-bromo cAMP/forskolin. Again, histamine required more oxyugen consumption per unit of intrinsic factor secretion.



FOR O.D. < 0.7:

P.U. = 94 * (O.D.) + 0.26

COR. COEF. = 0.9001

P << 0.01

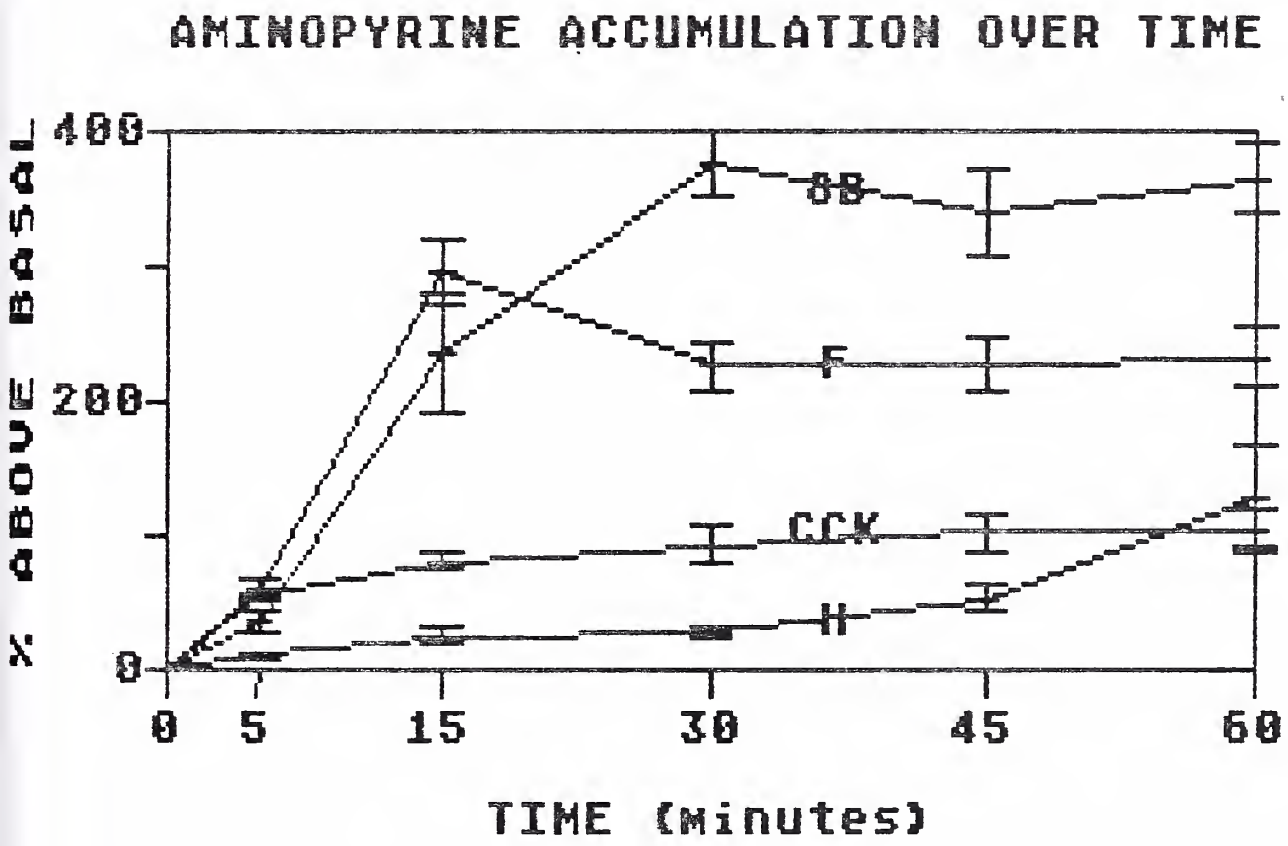
FOR O.D. > 0.7:

P.U. = 1100 * (O.D.) - 716

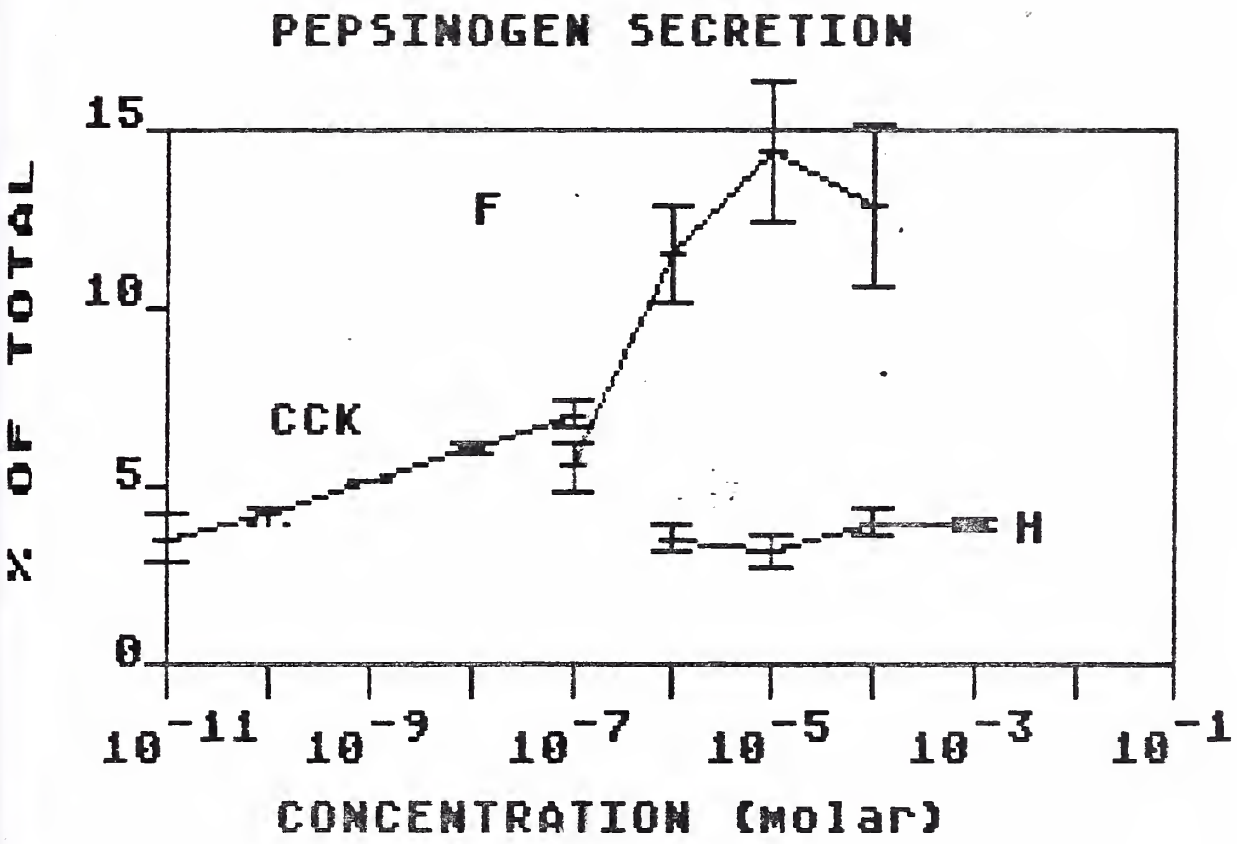
COR. COEF. = 0.917

P << 0.01

Figure 1. Pepsinogen Assay Standard Curve. Two straight lines are fitted to the hyperbolic relationship between optical density and peptic units to convert our data in optical density to peptic units.



ure 2. Aminopyrine accumulation over time. (8B-8 bromo cyclic AMP (10-4M), F-Forskolin, (10-4M), CCK-cholecystokinin (10-6M), and H-histamine (10-5M). Results are graphed as percent stimulated aminopyrine accumulation over unstimulated.



igure 3. Pepsinogen secretion in response to specific secretagogues (CCK-cholecystokinin, F-Forskolin, and H-histamine) CCK yields a linear dose response curve while F is linear over lower concentrations.

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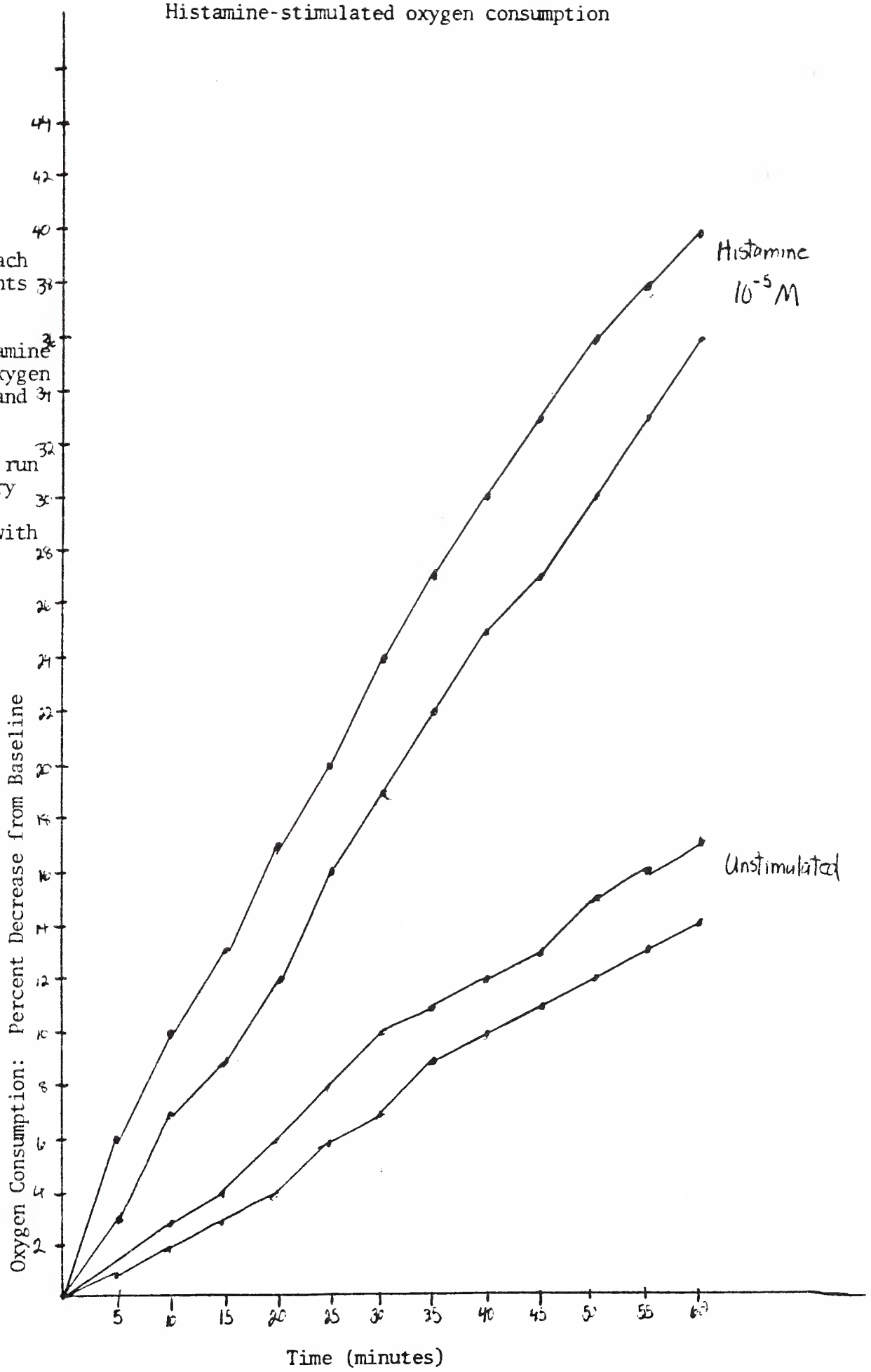

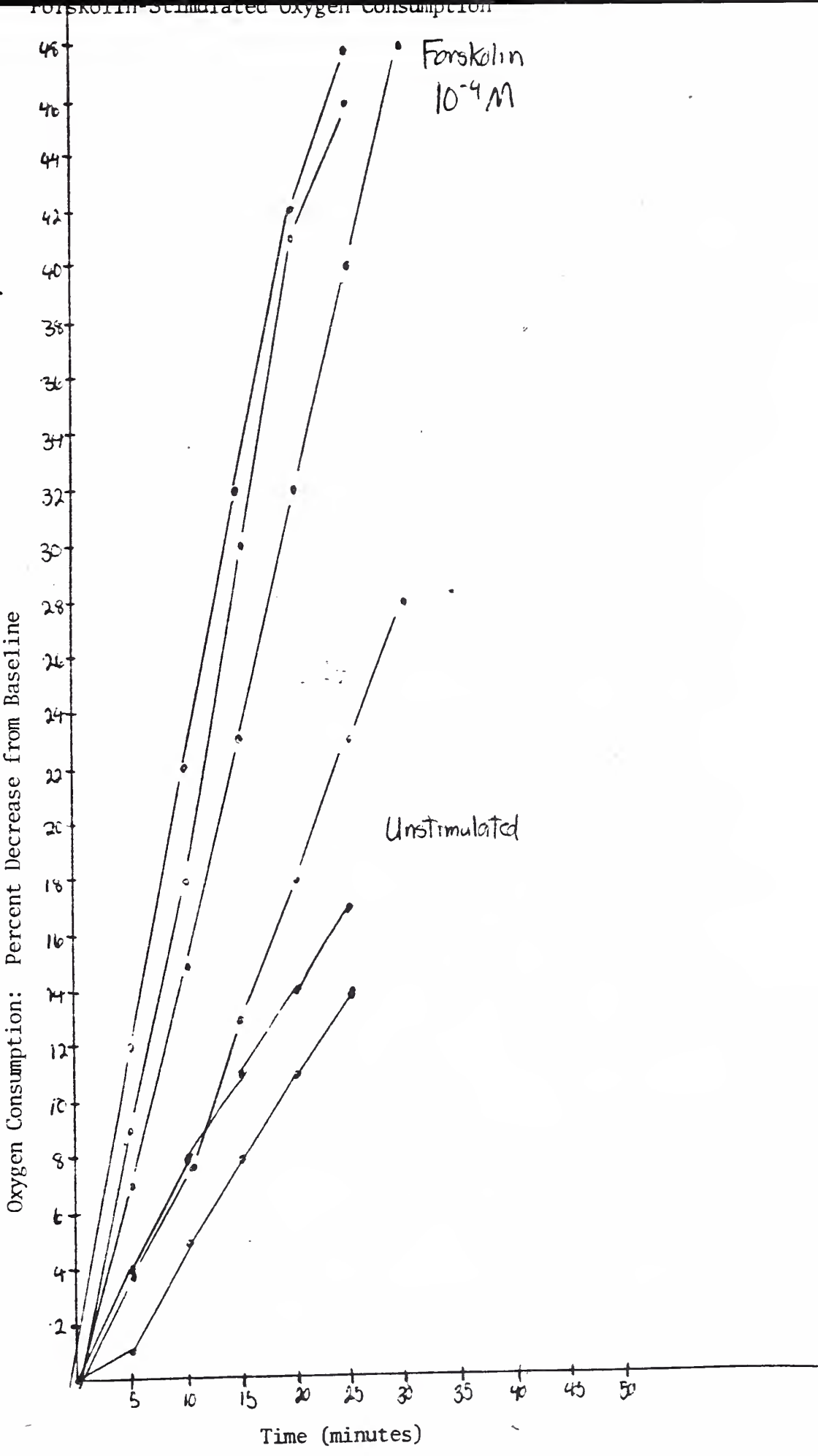

gure 5. Each line presents a single run. mparisons were made tween stimulated and stimulated oxygen nsumption.

gure 6. The
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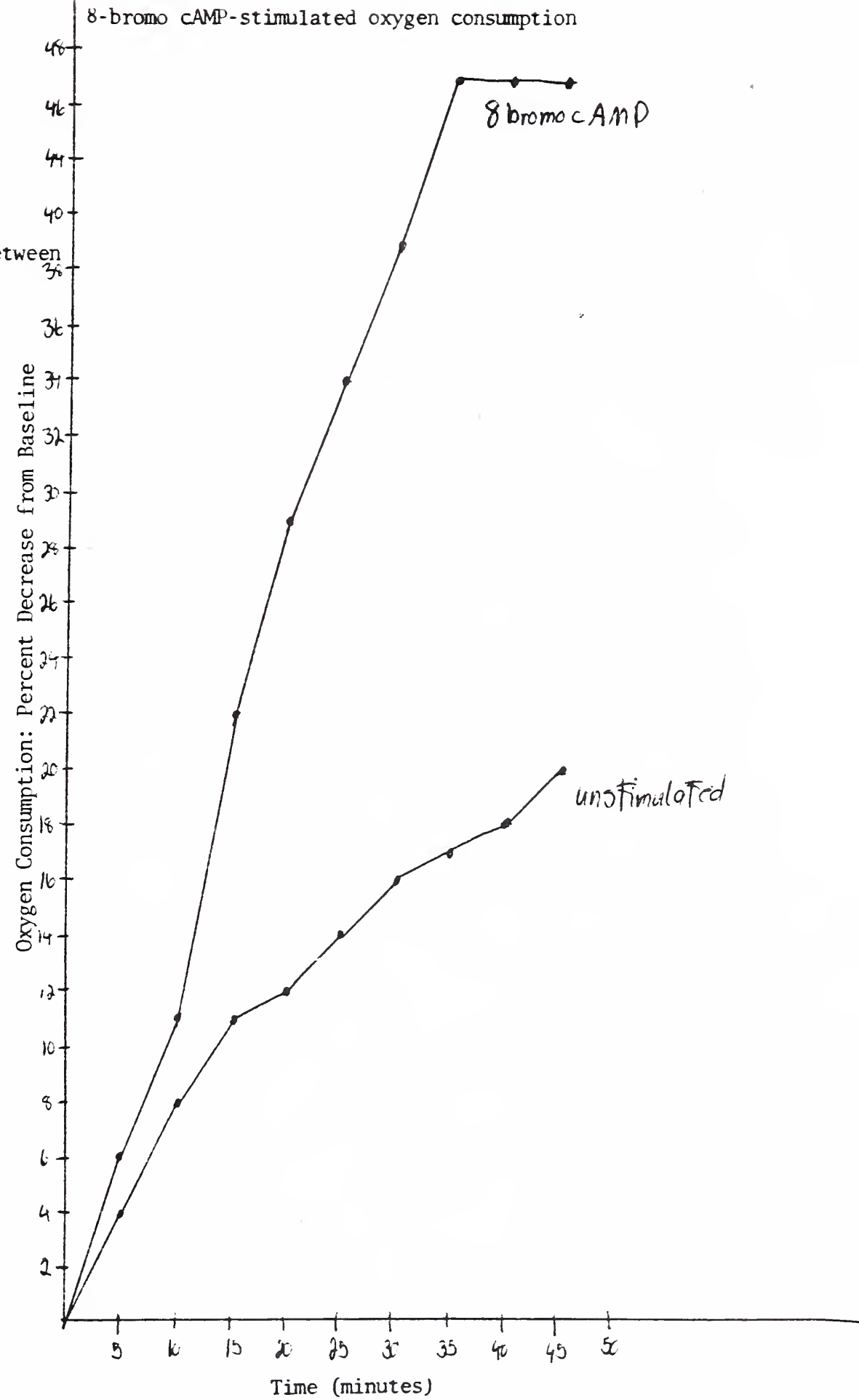


Table 1: Equations and Correlation Coefficients for Comparisons Among Secretagogues. "IF x AP" correlates to the graph with intrinsic factor secretion on the abscissa and amonopyrine accumulation on the ordinate. N/A indicates those combinations with too few points to generate a line.

	IF x AP	AP x O_2	IF x O_2
amine	y=0.0785x + 103.3 r=0.656	y=0.252x-17.5 r=0.763	y=0.0382x + 3.14 r=0.968
ecystokinin	y=0.692x + 93.6 r=0.664	N/A	N/A
omo cAMP	y=0.356x + 122 r=0.85	N/A	N/A
kolin	N/A	N/A	N/A
omo cAMP and Forskolin	y=0.315x + 160 r=0.751	y=0.05x - 4.09 r=0.807	y=0.0134x + 4.6 r=0.536

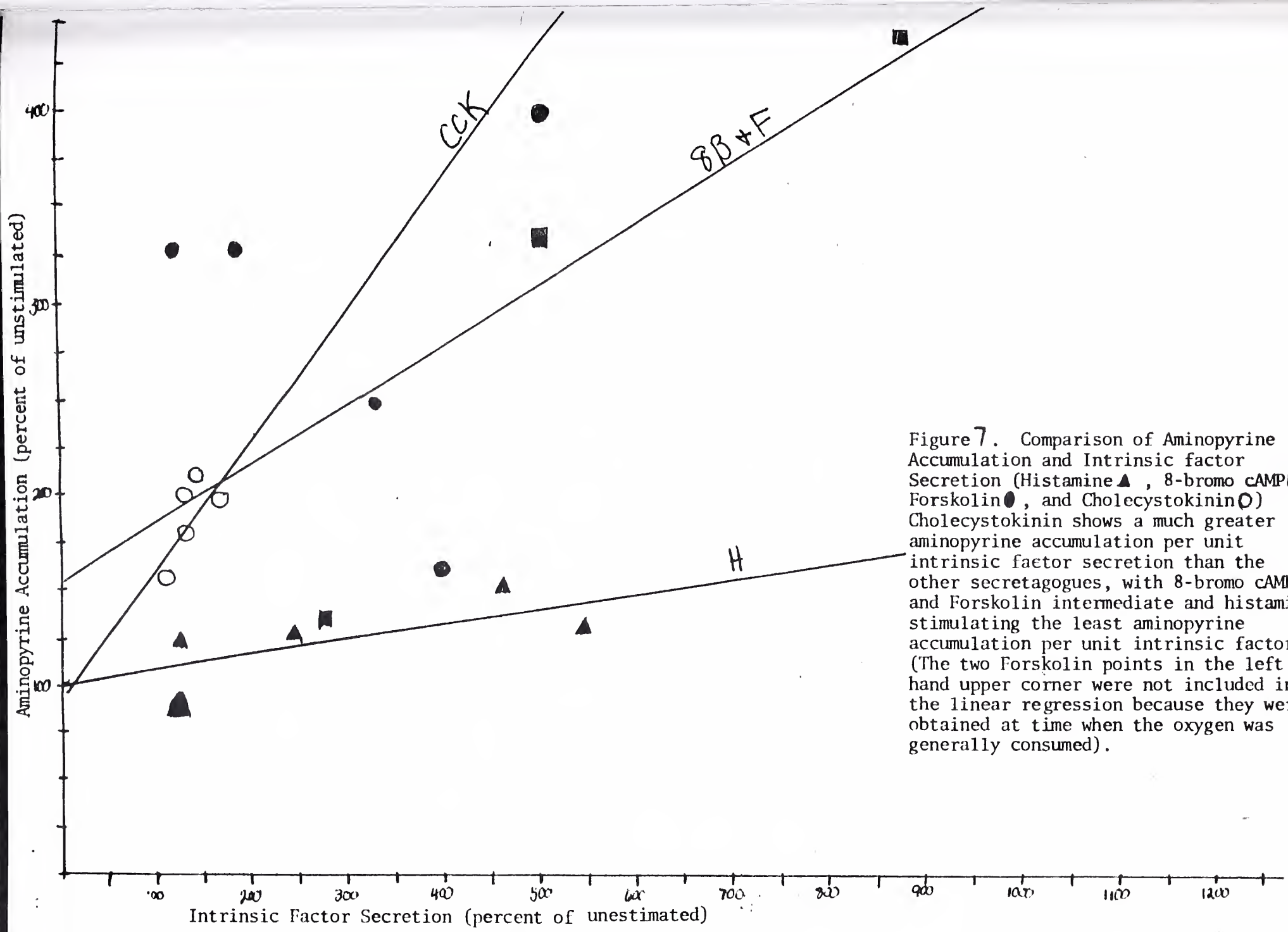


Figure 7. Comparison of Aminopyrine Accumulation and Intrinsic factor Secretion (Histamine ▲ , 8-bromo cAMP ■ Forskolin ● , and Cholecystokinin ○) Cholecystokinin shows a much greater aminopyrine accumulation per unit intrinsic factor secretion than the other secretagogues, with 8-bromo cAMP and Forskolin intermediate and histamine stimulating the least aminopyrine accumulation per unit intrinsic factor. (The two Forskolin points in the left hand upper corner were not included in the linear regression because they were obtained at time when the oxygen was generally consumed).

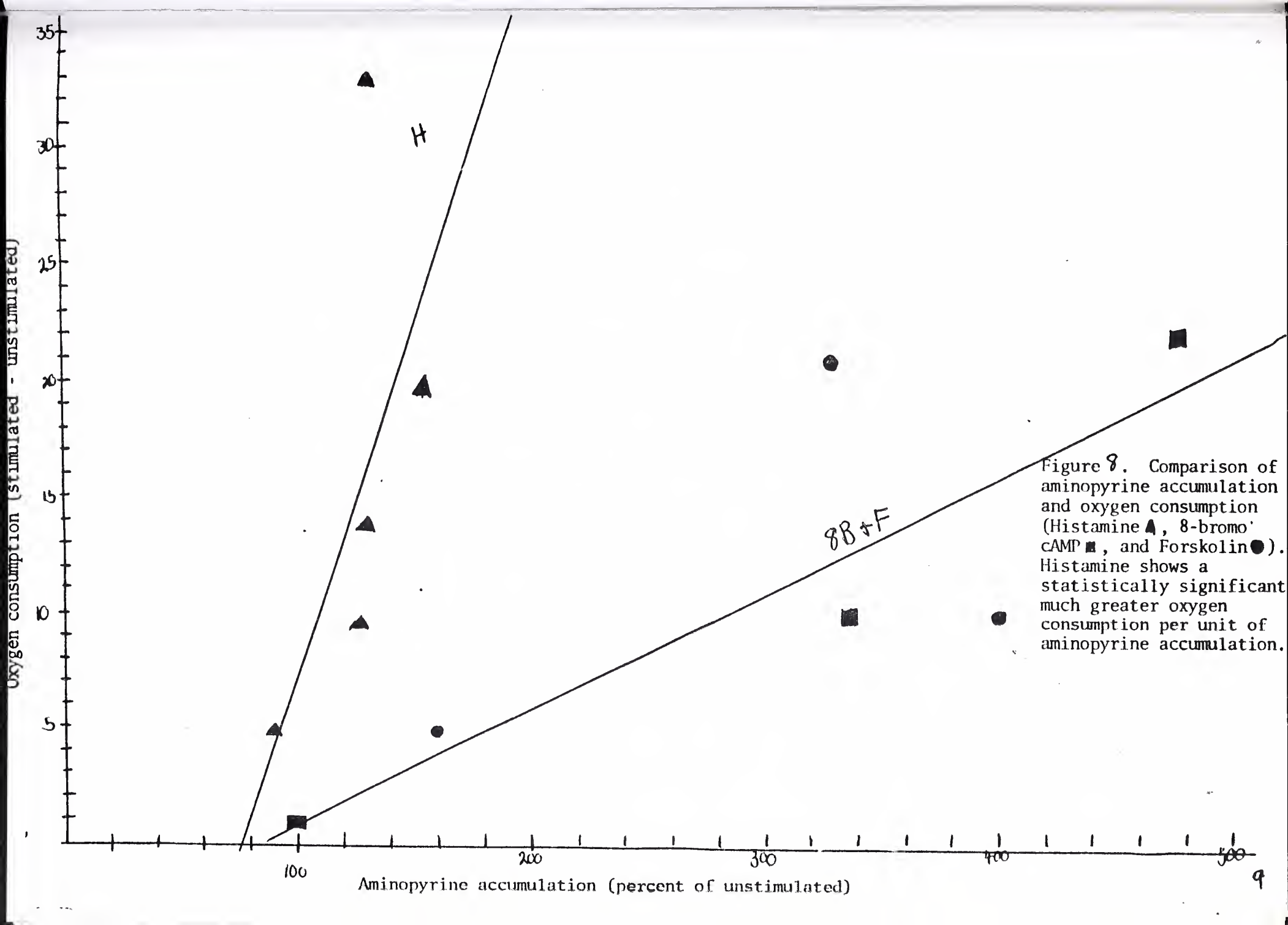


Figure 8. Comparison of aminopyrine accumulation and oxygen consumption (Histamine ▲, 8-bromo cAMP ■, and Forskolin ●). Histamine shows a statistically significant much greater oxygen consumption per unit of aminopyrine accumulation.

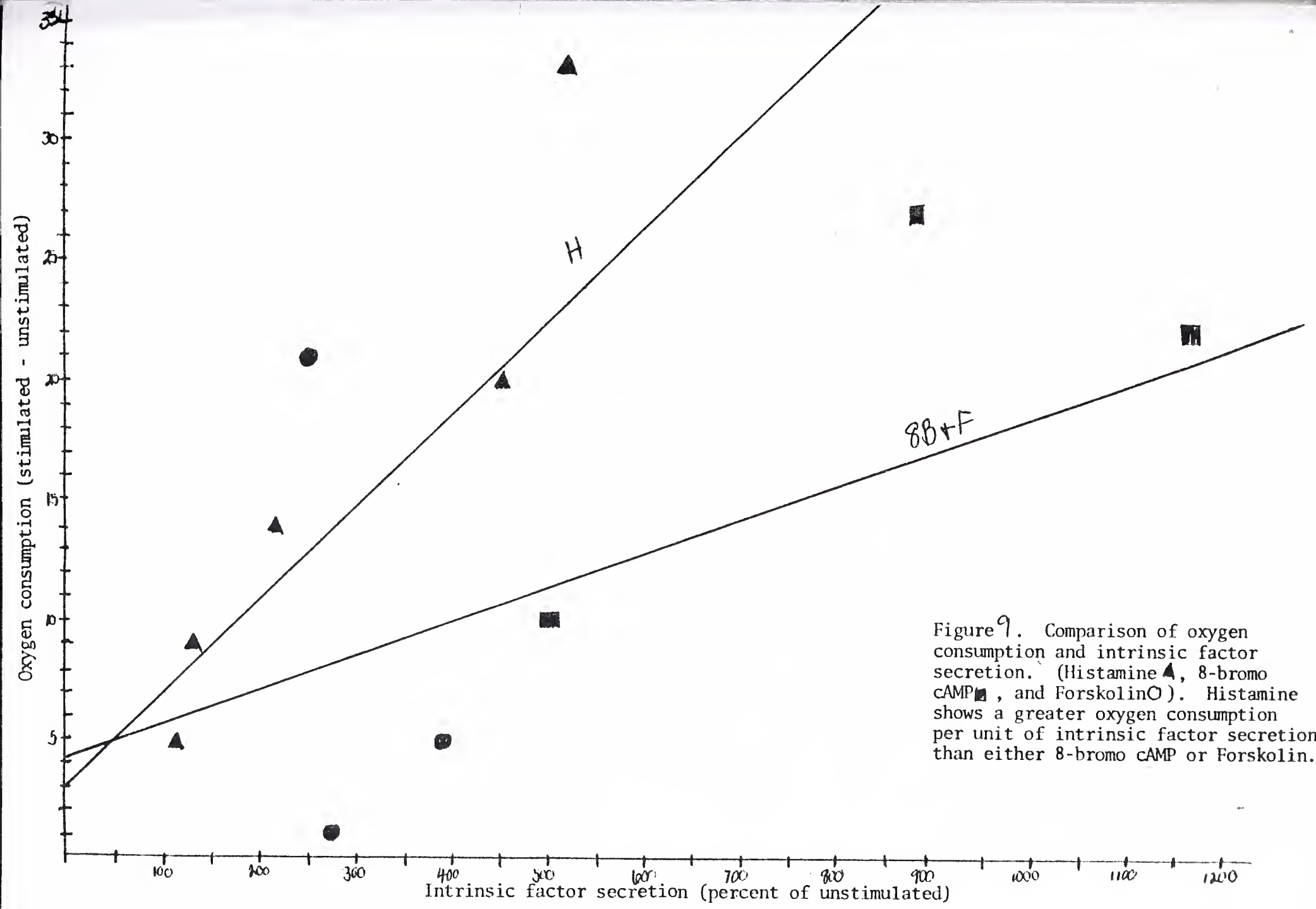
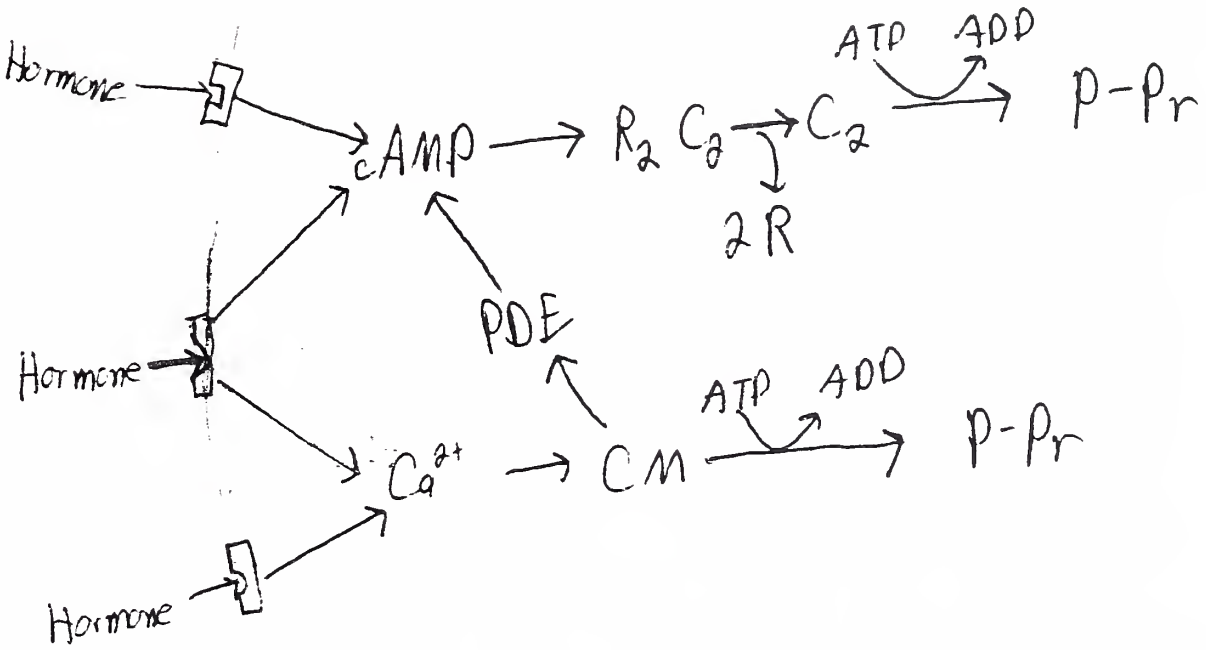


Figure 9. Comparison of oxygen consumption and intrinsic factor secretion. (Histamine ▲, 8-bromo cAMP ■, and Forskolin ○). Histamine shows a greater oxygen consumption per unit of intrinsic factor secretion than either 8-bromo cAMP or Forskolin.

ure 10

Cell plasmalemma



gulatory subunit

talytic subunit

- phosphorylated protein

calmodulin

- phosphodiesterase

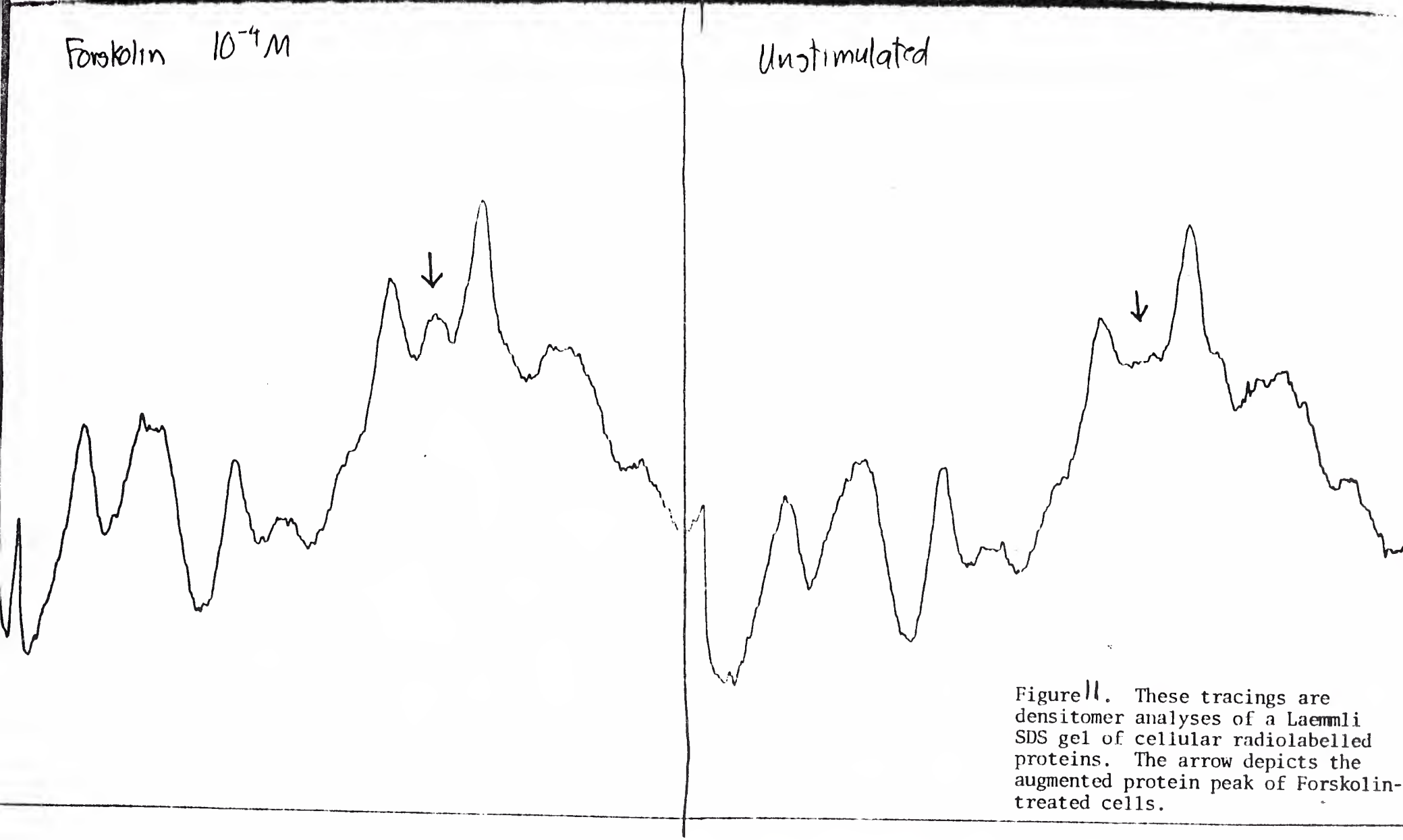


Figure 11. These tracings are densitomer analyses of a Laemmli SDS gel of cellular radiolabelled proteins. The arrow depicts the augmented protein peak of Forskolin-treated cells.

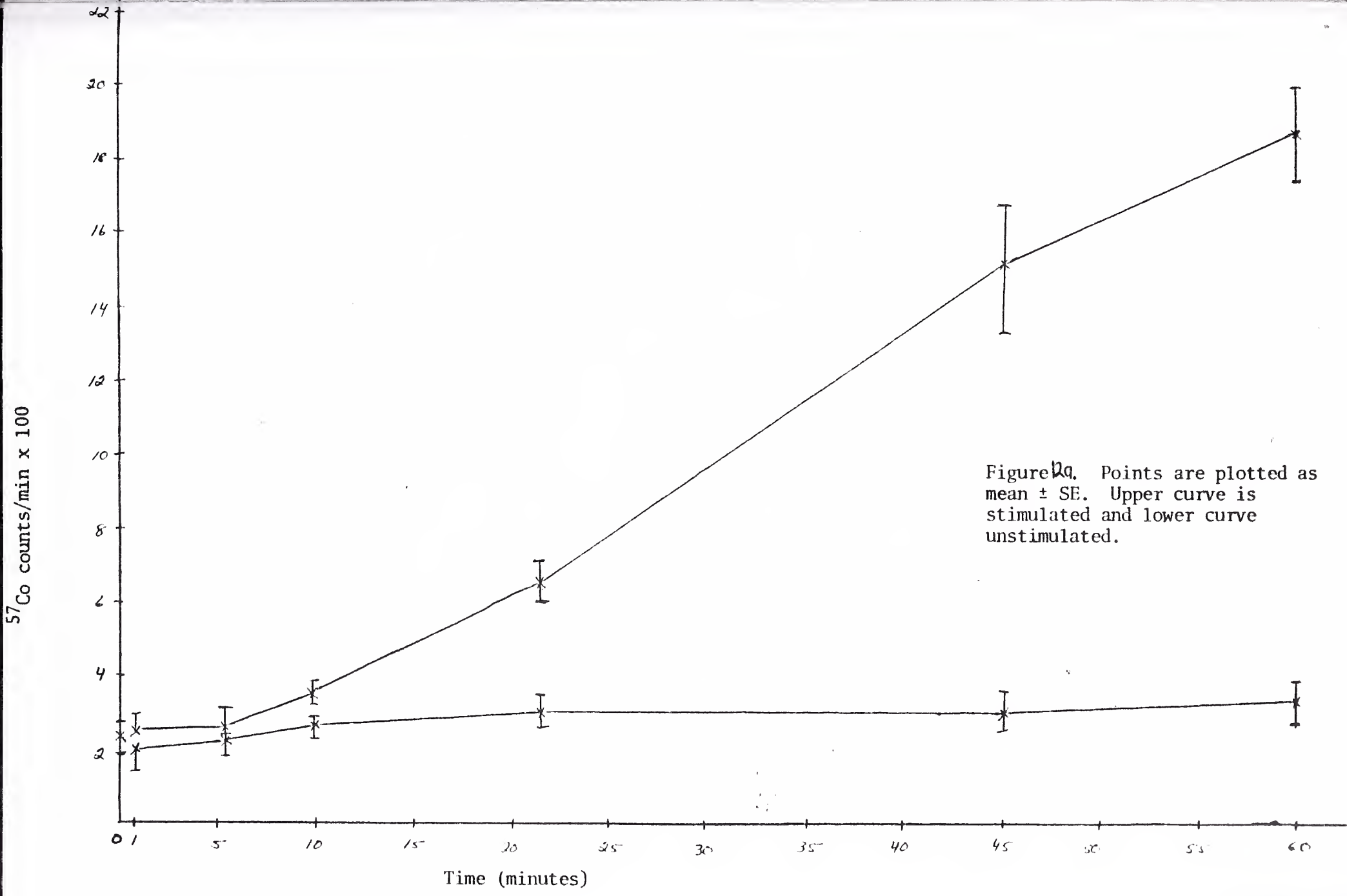


Figure 2a. Points are plotted as mean ± SE. Upper curve is stimulated and lower curve unstimulated.

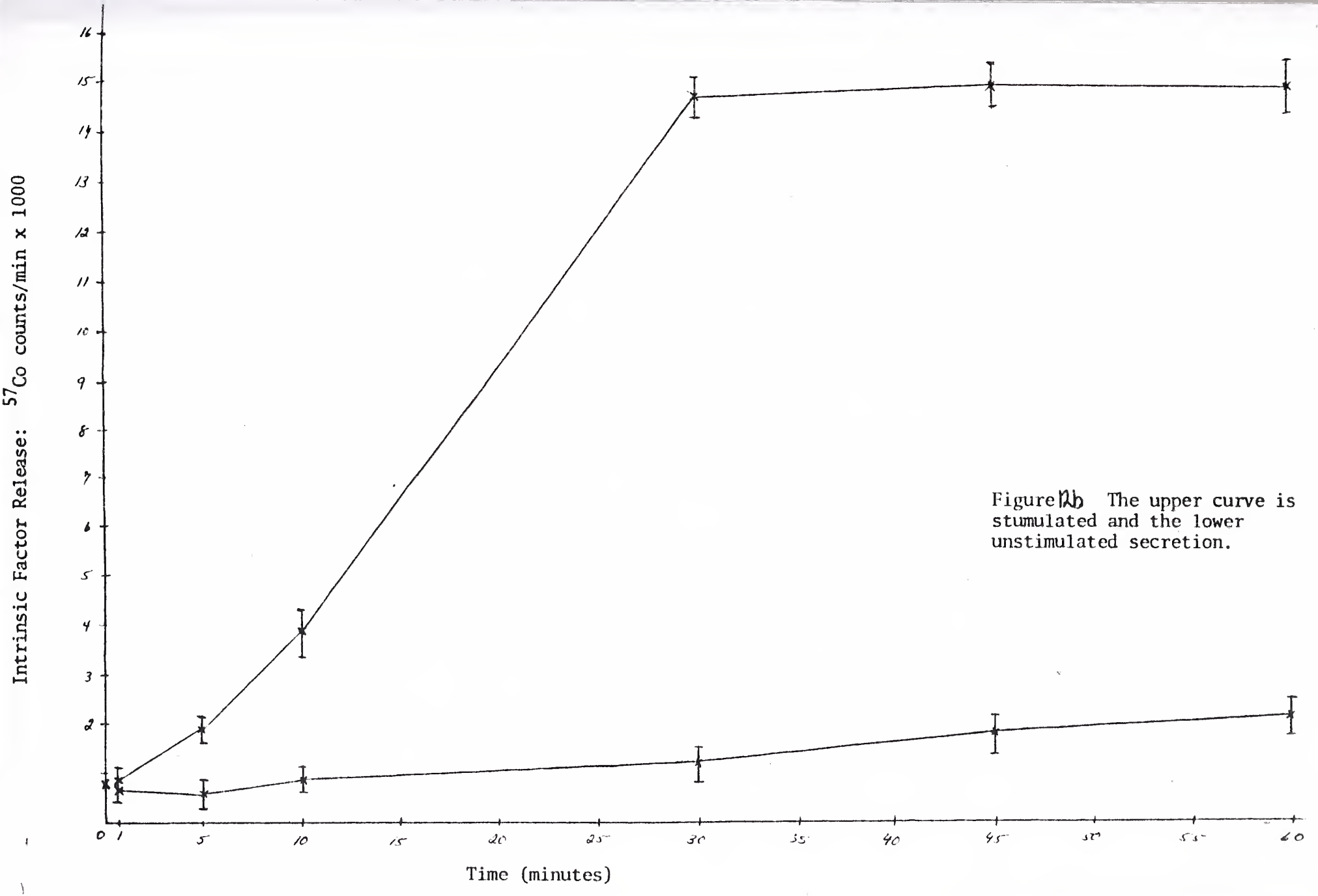


Figure 2b The upper curve is stumulated and the lower unstimulated secretion.

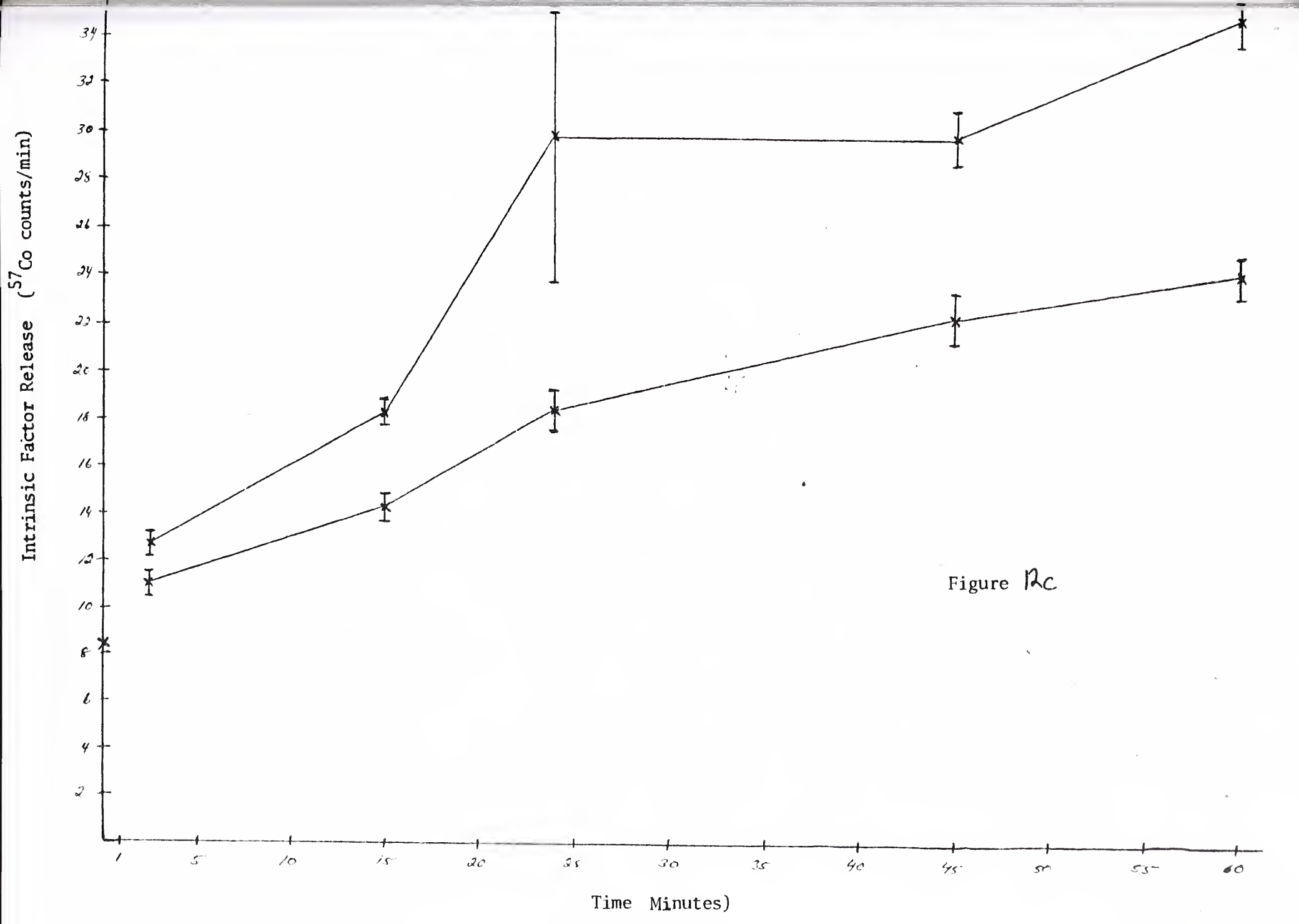

Intrinsic Factor Release (^{57}Co counts/min)
34
32
30
28
26
24
22
20
18
16
14
12
10
8
6
4
2
1
5
10
15
20
25
30
35
40
45
50
55
60
Time Minutes)

Figure 12c

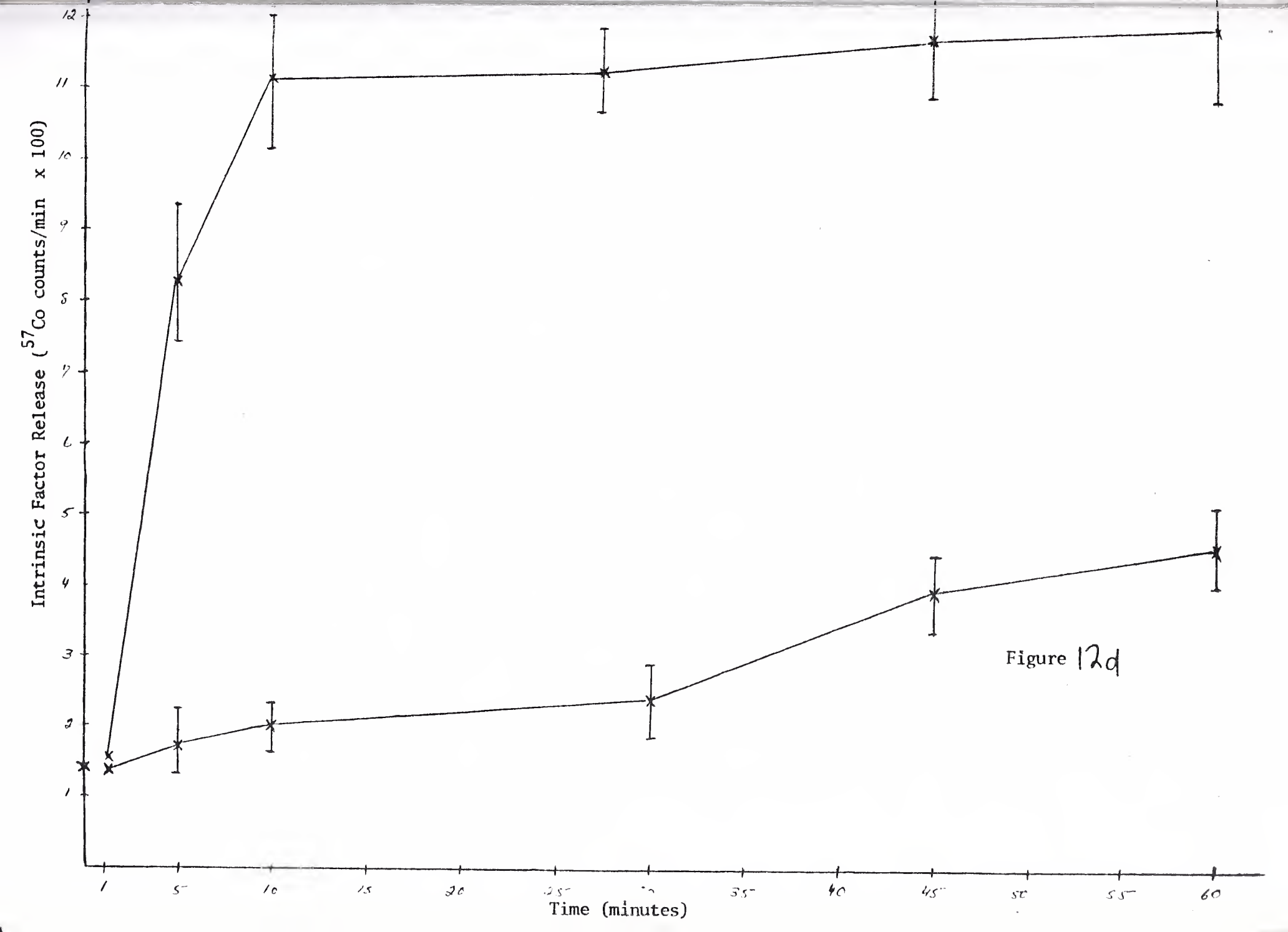

Intrinsic Factor Release (^{57}Co counts/min x 100)
Time (minutes)

Figure 12d

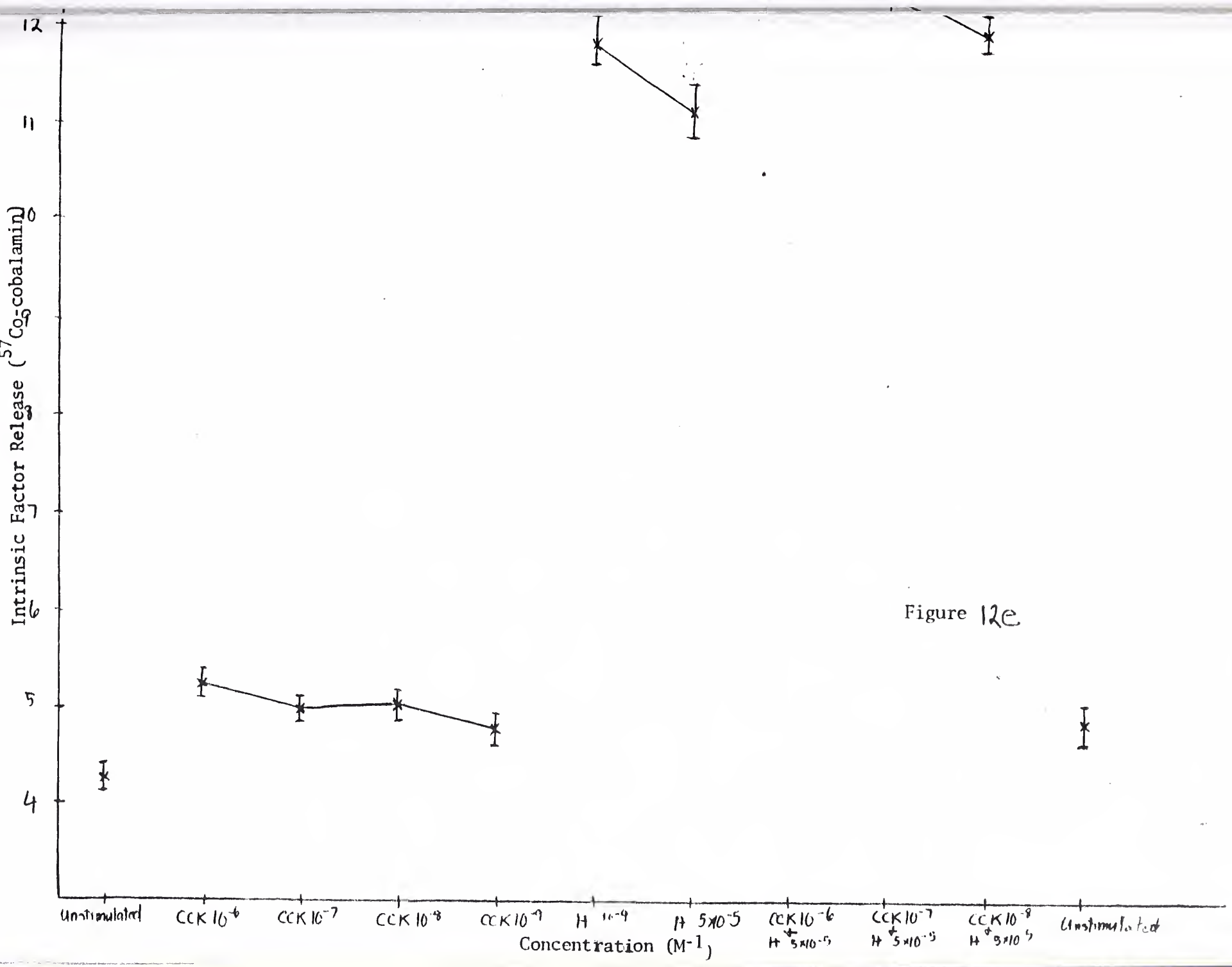

Intrinsic Factor Release (^{57}Co-cobalamin)
12
11
10
9
7
6
5
4
Unstimulated
CCK 10^{-6}
CCK 10^{-7}
CCK 10^{-8}
CCK 10^{-9}
H 10^{-4}
H 5×10^{-5}
CCK 10^{-6} + H 5×10^{-5}
CCK 10^{-7} + H 5×10^{-5}
CCK 10^{-8} + H 5×10^{-5}
Unstimulated
Concentration (M^{-1})

Figure 12e

DISCUSSION

The results of the studies undertaken have confirmed and extended previous reports of pepsinogen secretion in isolated gastric glands. We observed that cholecystokinin (CCK) at physiologic concentrations stimulated significant pepsinogen release from glands. This is comparable to previously reported data.[69,95,125] Since cholecystokinin has shown equivocal, if any, stimulation of pepsinogen secretion in intact subjects, it had been postulated that cholecystokinin might compete with gastrin for a specific receptor on the chief cell.[149,122,132] This suggestion was supported by the observation that CCK inhibits gastrin-stimulated pepsinogen secretion *in vivo*. This hypothesis, however, has not borne out by our studies in isolated gastric glands. In fact, the reverse was evident. Thus, CCK stimulated pepsinogen secretion while no stimulation of secretion by pentagastrin or human gastrin was measurable.[48] The location and nature of the receptor for these peptides is difficult to establish. Gastrin and CCK are extremely similar in structure so that competition for the same receptor is a possibility. Nonetheless, the reversal of their actions in pepsinogen secretion in going from intact subjects to isolated glands is difficult to explain, especially with the dearth of isolated gland studies. There are several possible explanations:

1. Either CCK or pentagastrin or both may be altered in *in vivo* studies by plasma proteases so that their binding to or activation of the receptor is changed.
2. The receptor, almost surely a protein, may be damaged by the collagenase digestion for the isolation of gastric glands.
3. The actual site of action of pentagastrin *in vivo* may be somewhere other than the glands.
4. An intermediate paracrine substance necessary for action/activation may be damaged in the preparation of glands.

Because some studies have shown inhibition of pentagastrin-stimulated pepsinogen secretion with H_2 receptor antagonists, it is possible that pentagastrin may act by release of endogenous histamine. Our preliminary data suggest that further studies, especially of isolated organ systems, are warranted to further elucidate this area.

Because CCK acts intracellularly in pancreatic acinar cells,[54,131] other authors have interpreted similar studies to mean that both calcium-calmodulin-mediated secretagogues (such as CCK) and cAMP-mediated secretagogues (such as histamine, forskolin and 8-bromo cAMP) may act together, synergistically, to stimulate pepsinogen secretion in isolated glands. Nonetheless, stimulation of the glands in calcium-free medium has yielded equivocal results and no exacting studies on the role of calcium in pepsinogen secretion have been attempted.

Forskolin, as might be expected of an intracellular cyclic AMP modulator, substantially stimulated pepsinogen secretion. The magnitude was as great as any reported.[125] This compound should presumably produce a pure cAMP response intracellularly and might therefore be a useful probe in studies with isolated cells or gastric glands. The pattern of response with a steep rise at the lower concentrations suggests a greater potency and efficacy for forskolin than the other secretagogues we tested.

Histamine evoked a gradual rise in pepsinogen secretion with log increases in secretagogue concentration. Histamine-stimulated pepsinogen secretion was not reported in vivo[137] nor in the single studies of gastric glands[97] nor canine mucosal cells.[8] Since the differences are small, it is possible that other studies may have failed to reach statistical significance due to lower concentration of gastric glands in each incubation. An alternative explanation might be that our glands were leaking pepsinogen due to chief cell death. However, the oxygen consumption studies indicate excellent viability. Furthermore, there are no reports which suggest that gland survival is a problem.

The largest percentage of total pepsinogen secretion reported to date is 18 percent with a combination of carbachol and secretin. Forskolin gave a 14 percent stimulation in our studies. It is, therefore, possible that only a small fraction of intracellular

pepsinogen is available for secretion, at least in response to a single stimulus. Alternatively, it may be that secretagogues present in the incubation medium are rapidly depleted a short while and that additional secretagogue would yield a larger percentage pepsinogen secretion. The differences are especially significant when compared to the much larger percentage of total intrinsic factor secreted in similar studies. These studies did not evaluate the total cellular pepsinogen remaining at the end of the experiment. Evaluation of the this factor might be relevant to establish whether glands synthesize pepsinogen at a rate comparable to their secretory status.

Intrinsic factor secretion has not been studied in gastric glands. Basically, our work has shown that both histamine and activators of intracellular cAMP stimulate significant secretion of at concentrations within physiologic limits. Our work shows significant basal secretion, as *in vivo*, that may be a property of the parietal cell or may be due to paracrine compounds released by the glands that stimulate intrinsic factor secretion. The signficant difference between the basal rates of intrinsic factor and pepsinogen secretion supports the consideration that the parietal cell has a high unstimulated intrinsic factor secretion.

Our work shows a small but significant stimulation of intrinsic factor secretion by cholecystokinin. Studies in dispersed cells in rat,[9] man,[9] and rabbit[134] indicated that activators of

intracellular cAMP stimulated intrinsic factor secretion in species (rabbit and human) in which parietal cells synthesized intrinsic factor. In addition, it was evident that carbachol and pentagastrin released intrinsic factor in rats in whom chief cells synthesize intrinsic factor. Our data convey evidence that the combination of histamine and cholecystokinin produce a synergistic stimulaton of intrinsic factor. Other studies in our laboratory demonstrated a significant stimulation of intrinsic factor secretion (129±4 percent) by pentagastrin at 10^{-7} M with synergistic stimulation by the addition of histamine at $5x10^{-5}$ M (277± 14 percent). This is the first demonstraton of intrinsic factor release in rabbit gastric glands by members of the pentagastrin family of peptides. Further comment will be detailed in subsequent discussion of intracellar messengers.

As alluded to in the Introduction, several studies in cells and glands have reported that secretagogues which stimulate intrinsic factor secretion in both glands and dispersed cells act through intracellular cAMP. The stimulation by CCK of intrinsic factor and its synergism with histamine are evidence that intracellular calcium can initiate or potentiate intrinsic factor secretion since CCK has been shown to act via intracellular calcium as a second messenger in amylase secretion by pancreatic acinar cells.[50,51]. As with pepsinogen, this must be confirmed with more specific studies.

The experiment utilizing aminopyrine accumulation and oxygen consumption support the observation that cAMP-mimetics are far more efficacious in the stimulation of acid secretion than are cholecystokinin or histamine. It is noteworthy that the accumulation of aminopyrine by 8-bromo cAMP or forskolin-stimulated glands may in fact have been hampered at the longer time periods by the rapid oxygen consumption. This occurred despite precautions to maintain maximal reoxygenation during stimulation. There are, however, a number of reservations in the interpretation of aminopyrine studies as a measurement of acid secretion. Cholecystokinin stimulated almost 100 percent aminopyrine accumulation above basal, suggesting the existence of a gland receptor for CCK that stimulates acid secretion. Chew and Hersey's finding of a direct gastrin-stimulation of acid secretion and our demonstration of CCK stimulation of acid secretion raise the possibility that the glands possess a receptor for this family of peptides. Of note is the difference between CCK and gastrin in the stimulation of pepsinogen by glands - CCK gives a strong stimulation while gastrin gives none at all in our work and several other studies. This suggests that gland receptors, possibly on chief cells, are capable of differentiating between the two hormones. The failure of CCK to stimulate acid secretion <u>in vivo</u> must raise the same questions as its failure to stimulate pepsinogen

secretion in vivo since it stimulates both pepsinogen and acid secretion by isolated gastric glands. As above, this is the first demonstration of CCK stimulated aminopyrine accumulation in isolated glands, indicating that CCK can stimulate acid, pepsinogen and intrinsic factor secretion in isolated glands.

The actions of histamine on each of the parameters: pepsinogen, intrinsic factor and acid secretion, was qualitatively different. For each parameter, histamine stimulated an almost exclusively linear secretion over time with no initial burst of secretion. This is especially surprising when compared to forskolin and 8-bromo cAMP since all three compounds are reputed to act via the same mechanism. It may be that at this dosage the two cyclic AMP-mimetics simply overwhelmed the capacity of the glands, and the data are not representative of a reasonable dose response curve. Another possibility is seldom considered in stimulus - secretion coupling - but require evaluation. Secretion is a combination of synthesis of the compound to be secreted and an "emptying" signal. One model for this emptying proposes that the magnitude of secretion is determined primarily by the density of granules near the secretory surface of the cell.[137] Thus, intracellular modulators may have either trophic, emptying, or both, actions on a substance to be secreted. Since one of the actions of gastrin is known to be trophic viz stimulation of cellular divi-

sion throughout the gastrointestinal tract it is possible that it or other secretagogues may also act to promote the synthesis of peptide hormones to be excreted.

Figure 10 is an illustration of the putative action and interaction of cAMP and calcium-calmodulin as expressed by Berglindh.[17] This model demonstrates how one hormone may activate both systems, presumably with synergistic actions. Phosphorylated proteins may, depending on the protein, be activated or inactivated by phosphorylation. The mechanisms depicted here allow for each system to be activated by a single hormone or for both to be activated by the same hormone. The latter has not been demonstrated in gastric cells. This model does not explain how final specificity of action may be differentiated between two hormones that activate the same system.

Figures 7 through 9 are graphical comparisons of the three parameters associated with parietal cells: oxygen consumption, intrinsic factor secretion, and aminopyrine accumulation. Two of the three are expressed on the abscissa and ordinate and the plotted point is the indicated secretagogue time point that gave the respective responses. The slope of these linear regressions delineate the relative changes in one parameter with respect to another. Such an analysis may give a due to hormone specificity of action for thse hormones that act via the same intracellular messengers.

Examination of the graphs reveals some interesting differences. 8-bromo cAMP and forskolin, despite differences in their stimulation of specific parameters, reveals great similarities in the relative stimulation of these parameters, i.e., intrinsic factor versus aminopyrine accumulation, aminopryine accumulation versus oxygen consumption and intrinsic factor versus oxygen consumption. In fact, the differences between the lines delineated by thir points are statistically insignificant. In contrast, histamine stimulates relatively much more intrinsic factor secretion than aminopyrine accumulation, indicating that the intracellular effect of histamine binding at the cellular surface may be more complex than simple activation of adenyl cyclase alone. Histamine's much greater oxygen consumption per unit of aminopyrine accumulation may be due to that amount of cellular energy required to synthesize and secrete intrinsic factor. Thus, in these cells and with histamine as a stimulus, oxygen consumption must be viewed as a relatively poor marker of acid secretion. This suggestion could be addressed by inhibition studies using an H_2 receptor antagonist or a H-K+ ATPase inhibitor. Cholecystokinin represents the other extreme in that it stimulates relatively much more aminopyrine accumulation per unit of intrinsic factor secretion than any of the other secretagogues. This might be interpreted to indicate that intracellular calcium has a greater effect on acid secretion than intrinsic factor secretion.

The lack of oxygen consumption data for CCK prevents further comparisons. In additon, the comlexity of interpretation of aminopyrine data under these experimental conditions limits the value of further hypothesis in this area. Particularly since it is probable that a vasolateral $Na\text{-}H^{+}$ pump may exist (personal communication with Professor N. Silon, visitor to the Gastrointestinal Surgical Research Group).

The mechanism for these differences is obscure except where individual secretagogues are believed to act via a different one of the two intracellular activator mechanisms. The contrast is especially interesting with histamine, whose line might be expected to merge with that of forskolin and 8-bromo cAMP because of the well characterized action of histamine on intracellular cAMP. Several possibilities exist to explain this difference:

1. Histamine may actually co-activate calcium channels in its binding to the cell membrane. This seems unlikely given its difference from cholecystokinin in aminopyrine accumulation relative to intrinsic factor secretion and the fact that the combination of CCK and histamine is synergistic in the stimulation of intrinsic factor secretion.
2. Histamine may activate as as yet unknown alternative second messenger system.

3. The actual concentration of intracellular cAMP may tend toward differential activation of specific catalytic subunits inside the cell since it is possible that different regulatory subunits would have different binding affinities for cAMP.

The elucidation of these alternatives will require substantial investigation of the intracellular mechanisms of stimulus - secretion coupling. We have, therefore, initiated in our laboratory studies of stimulus-dependent (specifically forskolin) protein phosphorylation in isolated gastric glands. Our work uses ^{32}P radiolabeled adenosine triphosphate incubations of isolated gastric glands and Laemmli SDS-polyacrylamide gel resolution of intracellular proteins. Preliminary results, as shown in Figure 11, indicate an enhancement of phosphrylated protein bands around 92 kilodaltons. It is possible that elucidation of these intracellular mechanisms will be the key to the further comprehension of the modulatory mechanisms of gastric secretion.

It is estimated that a number of persons per year in the United States suffer from acid-peptic disease. In the last quarter of a century, the identification of the control mechanism for acid secretion revolutionized the management of peptic ulcer disease. I believe that the elucidation of the cellular modulation of acid secretion is critical in the further development of rational therapeutic techniques and management of this common and ill understood disease process.

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